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Inventors: David P. Andrew, Brian A. Zabel and Paul D. Ponath
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METHODS OF IDENTIFYING AGENTS WHICH INHIBIT GPR-9-6

RELATED APPLICATIONS

This application is a divisional of Application No. 09/266,464, filed March 11, 1999.
The entire teachings of the above application are incorporated herein by reference.

BACKGROUND OF THE INVENTION

- Chemokines are a large and growing family of nearly forty 6-14 kD (non-
glycosylated) heparin binding proteins that mediate a wide range of biological functions
(Taub, D.D. and Openheim, J.J., *Ther. Immunol.*, 1:229-246 (1994)). The chemokines
can be divided into families based on the position of four cysteine residues that form
two disulfide bonds (Kelner, G.S., *et al.*, *Science*, 266:12395-1399 (1994); Bazan, J.F.,
et al., *Nature*, 385:640-644 (1997); Pin, Y., *et al.*, *Nature*, 385:611-617 (1997)).
Chemokine receptors can also be divided into families based on the type of chemokine
they bind, although, no clear structural differences have been identified that distinguish
the receptor sub-families (Mackay, C.R., *J. Exp. Med.*, 184:799-802 (1996)). In
addition, there are a number of so called "orphan" chemokine receptors (e.g., GPR-9-6)
which share sequence homology with well characterized chemokine receptors.
However, the biological functions and specific agonists of orphan receptors remain
unknown.

- Chemokines play a vital role in leukocyte adhesion and extravasation. For
example, in various *in vitro* assays, chemokines can induce the chemotaxis or
transendothelial migration of leukocytes (Taub, D.D. and Openheim, J.J., *Ther.*
Immunol., 1:229-246 (1994)), while *in vivo* injection (Taub, D.D., *et al.*, *J. Clin. Invest.*,

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97:1931-1941 (1996)) or over-expression of chemokines (Fuentes, M.E., *et al.*, *J. Immunol.*, 155:5769-5776 (1995)) can result in leukocyte accumulation at the site of chemokine injection or expression. Antagonists of chemokines can prevent leukocyte trafficking (Bargatze, R.F. and Butcher, E.C., *J. Exp. Med.*, 178:367-372 (1993)) and
 5 may have beneficial effects in several models of acute and chronic inflammation (Sekido, N., *et al.*, *Nature*, 365:654-657 (1993); Karpus, W.J., *et al.*, *J. Immunol.*, 155:5003-5010 (1995)). Chemokines have also been reported to modulate angiogenesis (Gupta, S.K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 92:7799-7803 (1995)), hematopoiesis (Taub, D.D. and Openheim, J.J., *Ther. Immunol.*, 1:229-246 (1994)) as well as T
 10 lymphocyte activation (Zhou, Z., *et al.*, *J. Immunol.* 151:4333-4341 (1993); Taub, D.D., *et al.*, *J. Immunol.*, 156:2095-2103 (1996)). In addition, several chemokine receptors act as co-receptors, along with CD4, for entry of M tropic and T tropic HIV-1 (Choe, H., *et al.*, *Cell*, 85:1135-1148 (1996); Feng, Y., *et al.*, *Science*, 272:872-877 (1996)).

Several subsets of CD4 lymphocytes can be defined based on their expression of
 15 various adhesion molecules that are known to effect trafficking to different physiologic sites (Mackay, C.R., *Curr. Opin. Immunol.*, 5:423-427 (1993)). For example, CLA⁺ memory CD4 lymphocytes traffic to the skin (Berg, E.L., *et al.*, *Nature*, 174(6):1461-1466 (1991)), while CLA⁻ $\alpha 4\beta 7^{+}$ memory CD4 lymphocytes traffic to mucosal sites (Hamman, A., *et al.*, *J. Immunol.*, 152:3282-3292 (1994)). Leukocyte adhesion to
 20 endothelium is thought to involve several overlapping steps including rolling, activation and arrest. Rolling leukocytes are exposed to factors expressed at the adhesion site resulting in activation of the leukocyte and up-regulation of integrin-mediated adhesion. As a consequence of such integrin-mediated interactions, leukocytes arrest on the endothelium (Bargatze, R.F. and Butcher, E.C., *J. Exp. Med.*, 178:367-372 (1993);
 25 Bargatze, R.F., *et al.*, *Immunity*, 3:99-108 (1995)). Leukocyte activation and up-regulation of integrin molecules occurs via a pertussis toxin sensitive mechanism that is thought to involve chemokine receptors (Bargatze, R.F. and Butcher, E.C., *J. Exp. Med.*, 178:367-372 (1993); Campbell, J.J., *et al.*, *Science*, 279:381-383 (1998)).

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Memory CD4⁺ lymphocytes can be grouped based upon the expression of certain chemokine receptors. For example, CXCR3, CCR2 and CCR5 (Qin, S., *et al.*, *Eur. J. Immunol.*, 26:640-647 (1996); Qin, S., *et al.*, *J. Clin. Invest.*, 101:746-754 (1998); Liao, F., *et al.*, *J. Immunol.*, 162:186-194 (1999)) are all expressed on subsets of
5 memory CD4 lymphocytes, and certain chemokines act selectively on naive T cells (Adema, G.J., *et al.*, *Nature*, 387:713-717 (1997)). Furthermore, several chemokines which are ligands for such receptors have been shown to be expressed in inflammatory sites (Gonzalo, J.A., *et al.*, *J. Clin. Invest.*, 98:2332-2345 (1996)) and in some cases in lymph nodes draining a challenged site (Tedla, N., *et al.*, *J. Immunol.*, 161:5663-5672
10 (1998)). *In vitro* derived T_H1/T_H2 lymphocyte lines have also been shown to differentially express chemokine receptors. Specifically, T_H1 lymphocytes have been shown to selectively express CXCR3 and CCR5, while T_H2 lymphocytes selectively express CCR4, CCR8 and CCR3 (Bonecchi, R.G., *et al.*, *J. Exp. Med.*, 187:129-134 (1998); Sallusto, F.D., *et al.*, *J. Exp. Med.*, 187:875-883 (1998); Sallusto, F., *Science*,
15 277:2005-2007 (1997); Andrew, D.P., *et al.*, *J. Immunol* 161:5027-5038 (1998); Zingoni, A., *et al.*, *J. Immunol.*, 161:547-555 (1998)). Interestingly, in some cases the chemokines for these respective chemokine receptors, such as MDC for CCR4 and IP-10 for CXCR3, are induced by cytokines associated with a T_H1/T_H2 environment (Andrew, D.P., *et al.*, *J. Immunol* 161:5027-5038(1998); Luster, A.D., *et al.*, *Nature*,
20 315:672-676 (1985)).

SUMMARY OF THE INVENTION

The invention relates to an antibody (immunoglobulin) or functional fragment thereof (e.g., an antigen-binding fragment) which binds to a mammalian GPR-9-6 or portion of the receptor. In one embodiment, the antibody or antigen-binding fragment
25 thereof binds to human GPR-9-6. In another embodiment, the antibody or antigen-binding fragment thereof can inhibit the binding of a ligand to a mammalian GPR-9-6. In a preferred embodiment, the antibody or antibody-binding fragment can bind to human GPR-9-6 and inhibit the binding of TECK to the receptor.

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In another embodiment, the antibody or antigen-binding fragment of the invention binds to an epitope which is the same as or is similar to the epitope recognized by mAb 3C3 or an antigen-binding fragment thereof. For example, the binding of the antibody or antigen-binding fragment of the invention to human GPR-9-6 can be inhibited a peptide that consists of the amino acid sequence of SEQ ID NO:3. In another embodiment, the binding of the antibody or antigen-binding fragment of the invention to human GPR-9-6 can be inhibited by mAb 3C3. In a preferred embodiment, the antibody is mAb 3C3 or antigen-binding fragment thereof.

The invention also relates to an isolated cell that produces an antibody or antigen-binding fragment of the present invention, including those which bind to mammalian GPR-9-6 and inhibit the binding of a ligand to the receptor. In a particular embodiment, the isolated cell is murine hybridoma 3C3 (also referred to as murine hybridoma LS129-3C3-E3-1) deposited under ATCC Accession No. _____.

The invention also relates to a method of detecting or identifying an agent (i.e., molecule or compound) which binds to a mammalian GPR-9-6. In one embodiment, an agent which can bind to mammalian GPR-9-6 and inhibit (reduce or prevent) the binding of a ligand (e.g., TECK) to GPR-9-6 is identified in a competitive binding assay. In other embodiments, agents for use in therapy are identified in a direct binding assay. Thus, the invention encompasses methods of identifying agents which modulate GPR-9-6 function, such as, ligands or other substances which bind a mammalian GPR-9-6, including inhibitors (e.g., antagonists) or promoters (e.g., agonists) of receptor function. A suitable source of a mammalian GPR-9-6 or a ligand-binding variant thereof can be used to identify a GPR-9-6 binding agent in accordance with the method of the invention. In one embodiment, a cell (e.g., cell line, recombinant cell) that expresses a mammalian GPR-9-6 or a ligand binding variant thereof is used. In another embodiment, a membrane preparation of a cell that expresses a mammalian GPR-9-6 or a ligand binding variant thereof is used.

The invention also relates to therapeutic methods in which agents which can bind to a mammalian GPR-9-6 and modulate (inhibit or promote) a GPR-9-6 function

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are administered to a subject in need of such therapy. In one embodiment, the therapeutic method is a method of treating a subject having an inflammatory disease. In a preferred embodiment, the subject has an inflammatory diseases associated with mucosal tissues, such as an inflammatory bowel disease. In a particular embodiment, the inflammatory bowel disease is Crohn's disease or colitis. In another embodiment, the therapeutic method is a method of inhibiting GPR-9-6-mediated homing of leukocytes. In another embodiment, the method is a method of modulating a GPR-9-6 function.

The invention further relates to a method for detecting or quantifying a mammalian GPR-9-6 or a portion thereof in a biological sample. The method comprises contacting a biological sample and an anti-GPR-9-6 antibody or antigen-binding fragment of the invention under conditions suitable for binding, and detecting a complex formed between GPR-9-6 and the antibody or antigen-binding fragment. In one embodiment the biological sample comprises human cells or a fraction of said cells (e.g., membrane preparation).

The invention also relates to a test kit for identifying or quantifying a mammalian GPR-9-6 or a portion thereof in a biological sample. In one embodiment, the kit comprises an antibody of the invention and suitable ancillary reagents.

The present invention further relates to an antibody, antigen-binding fragment or agent as described herein for use in therapy (including prophylaxis) or diagnosis, and to the use of such an antibody, antigen-binding fragment or agent for the manufacture of a medicament for the treatment of a particular disease or condition as described herein (e.g., an inflammatory disease associated with mucosal tissues).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a dendrogram illustrating the relationship of GPR-9-6 to other leukocyte chemokine receptors. Using a clustal alignment analysis program (DNASTar), the protein sequences of leukocyte chemokine receptors were aligned and used to

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determine the phylogenetic distances between GPR-9-6 and several chemokine receptors.

Figures 2A-2B illustrate the specific binding of mAb 3C3 bind to GPR-9-6 transfectants. In Figure 2B, GPR-9-6/L1.2 transfectants were stained with mAb 3C3 (solid profile), anti-CCR6 antibody (.....) or with a murine IgG2b mAb (----) (n=2). In Figure 2B, CCR6/L1.2 transfectants were stained with mAb 3C3 (.....), anti-CCR6 antibody (solid profile) or with a murine IgG2b mAb (----) (n=2).

Figure 3 is a series of fluorescence plots which illustrate that GPR-9-6 is expressed on B lymphocytes and subsets of CD4 and CD8 lymphocytes. mAb 3C3 was used in two color studies on mononuclear cells along with anti-CD4 FITC, anti-CD8 FITC, anti-CD19 FITC, anti-CCR3 FITC and anti-CD56 Cychrome. For thymocytes, two color studies were performed with mAb 3C3 and anti-TcR Cychrome. GPR-9-6 expression on monocytes, eosinophils and neutrophils was evaluated in one color studies using isolated populations of these cells and mAb 3C3 (—) and Ig2b control (---). Anti-CCR2, anti-CCR3 and anti-CXCR2 antibodies were used as positive controls for monocytes, eosinophils and neutrophils, respectively (solid profiles) (n=3).

Figures 4A-4H are plots illustrating that GPR-9-6 is not expressed on immature dendritic cells (IMDC), mature dendritic cells (MDC) or T_H1/T_H2 lymphocytes. Mature (—) and immature dendritic cells (solid profile) were stained with anti-CCR5 (Figure 4A), anti-CD83 (Figure 4B), anti-CD86 (Figure 4C) or anti-GPR-9-6 (Figure 4D). Staining with IgG2b control on IMDCs (.....) is also shown. Figure 4E shows staining of umbilical CD4 lymphocytes with anti-CXCR4 (solid profile), anti-GPR-9-6 (—) and IgG2b (.....). Figures 4F-4H show staining of T_H1 (solid profiles) and T_H2 (—) lymphocytes with anti-CXCR3 (Figure 4F), anti- $\alpha4\beta7$ (Act1) (Figure 4G) or anti-GPR-9-6 (mAb 3C3) (Figure 4H) as indicated, with (.....) representing staining with an IgG2b control on T_H1 lymphocytes (n=3).

Figures 5A-5C are graphs illustrating the modulation of GPR-9-6 on lymphocytes over time and upon T lymphocyte activation. Mononuclear cells were isolated from one individual at set times over 14 days and stained in two color

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experiments using mAb 3C3 and anti-CD4 FITC or anti-CD19 FITC to examine GPR-9-6 expression on B and CD4 lymphocytes (Figure 5A). In Figures 5B-5C, mononuclear cells were activated with plate bound anti-TcR mAb OKT3 for 4 days, followed by expansion with IL-2 at 5 ng/ml. Aliquots of cells were stained over time with mAb 3C3 (Figure 5B) to determine GPR-9-6 expression upon T lymphocyte activation, or with anti-CCR6 mAb and anti-CCR5 mAb (Figure 5C) to determine expression of CCR-3 and CCR-5 upon T cell activation. (n=2)

Figure 6 is a series of fluorescence plots illustrating that GPR-9-6 is expressed on $\alpha 4\beta 7^{\text{high}}$ CLA^{-ve} CD4⁺ memory lymphocytes. Mononuclear cells were stained in three color experiments using anti-CD4 cychrome to gate on CD4 lymphocytes. The cells were also stained with anti-GPR-9-6 mAb 3C3 followed by F(ab')₂ anti-mouse IgG phycoerythrin to study GPR-9-6 expression on subsets defined with anti- α E (HML1, Beckman Coulter, Inc., Fullerton, CA), anti- β 7 (Fib504, PharMingen, San Diego, CA), anti-CD49d (HP2/1, PharMingen, San Diego, CA), anti-CLA (HECA 452, PharMingen, San Diego, CA), anti-CD45RO (UCLH1, PharMingen, San Diego, CA) and anti-CD62L (CD56)(PharMingen, San Diego, CA) (n=5).

Figure 7 is a series of fluorescence plots illustrating the expression of GPR-9-6 on CD4 lymphocytes in relation to other chemokine receptors. Mononuclear cells were stained in three-color experiments using anti-CD4 cychrome to gate on CD4 lymphocytes. The cells were also stained with anti-GPR-9-6 mAb 3C3 followed by F(ab')₂ anti-mouse IgG coupled to phycoerythrin to study GPR-9-6 expression on subsets defined with anti-CCR2 (R&D Systems, Minneapolis, MN), anti-CCR5 (PharMingen, San Diego, CA), anti-CCR6 (R&D Systems, Minneapolis, MN), anti-CXCR3 (1C6, Leukosite, Inc., Cambridge, MA), anti-CXCR4 (PharMingen, San Diego, CA) and anti-CXCR5 (R&D Systems, Minneapolis, MN), all of which were coupled to phycoerythrin (n=2).

Figures 8A-8F are a graph and series of histograms illustrating that GPR-9-6 is a chemokine receptor for TECK. GPR-9-6/L1.2 transfectants were tested for a chemotactic response to 10 to 1000 nM TECK (Figure 8A). Figure 8B shows that anti-

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(Figure 11B), and CD4 lymphocytes were assayed using 100 nM TARC (Figure 11C). In all assays, TECK-induced chemotaxis was inhibited by anti-GPR-9-6 (mAb 3C3). Irrelevant mAb anti-CCR6 mAb 2A9 (Figure 11A) and anti-CCR4 mAb 2B10 (Figure 11B) were also examined for their effect on CD4 or thymocyte chemotaxis to TECK or TARC. For CD4 lymphocyte chemotaxis the effect of mAb 3C3 on TARC induced CD4 lymphocyte chemotaxis was also tested (Figure 11C) as a further negative control (n=2).

Figures 12A-12C illustrate the tissue distribution of TECK and GPR-9-6. Multi-tissue Northern blot analysis filters (2 μ g RNA/lane) (ClonTech) and a Northern blot prepared using RNA from various cell lines (20 μ g/lane) were probed with 32 P TECK DNA probes (Figure 12A) or labeled GPR-9-6 (Figure 12B) to determine their tissue distribution. In Figure 12C, cDNA (ClonTech) from colon, small intestine, brain, lymph node, spleen, thymus, and genomic DNA were amplified in PCR (30 cycles) using primers designed from the sequence of GPR-9-6.

Figures 13A-13B are histograms illustrating that only $\alpha 4\beta 7^{\text{high}}$ CD4 and CD8 lymphocytes migrate to TECK. In a 4 color sort, memory CD8 lymphocytes defined by intermediate/negative expression of CD45RA and expression of CD27 and CD8 were sorted into $\alpha 4\beta 7$ negative, intermediate and high populations using Act1-phycoerythrin. For CD4 lymphocytes, memory CD4 lymphocytes defined by lack of CD45RA and expression of CD4 were sorted into $\alpha 4\beta 7^{-\text{ve}}$ CLA $^{-\text{ve}}$, $\alpha 4\beta 7^{-\text{ve}}$ CLA $^{+\text{ve}}$, and $\alpha 4\beta 7^{+\text{ve}}$ CLA $^{-\text{ve}}$ sub-populations based on CLA and $\alpha 4\beta 7$ expression using the anti- $\alpha 4\beta 7$ antibody Act1-Phyorythrin and the anti-CLA antibody HECA 452-FITC. These sub-populations of memory CD4 (Figure 13A) and CD8 (Figure 13B) lymphocytes were then examined for their ability to chemotax to 1 μ M TECK (n=2).

Figure 14A-14B illustrates a nucleotide sequence encoding human (*Homo sapiens*) GPR-9-6 (SEQ ID NO:1) deposited in Genbank under Accession Number U45982, having an open-reading frame beginning at position 58.

Figure 15 illustrates the amino acid sequence of a human GPR-9-6 protein (SEQ ID NO:2) encoded by the DNA sequence shown in Figure 14A-14B (SEQ ID NO:1).

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DETAILED DESCRIPTION OF THE INVENTION

Abbreviations used herein include: ECV304, human umbilical vein endothelial cell line (ATCC Accession No. CRL-1998); ADEC, adenoid expressed chemokine; IP10, IFN-gamma-inducible 10 kDa protein; IMDC, immature dendritic cell; I-TAC, 5 interferon-inducible T cell alpha chemoattractant; MCP-1, monocyte chemoattractant protein; SDF, stromal cell derived factor; MDC, mature dendritic cell chemokine; MIG, monokine induced by interferon-gamma; RANTES, regulated on activation normal T cell expressed; MIP3, macrophage inflammatory protein 3; MIP4, macrophage inflammatory protein 4; TECK, thymus expressed chemokine; SLC, secondary 10 lymphoid-tissue chemokine; DC, dendritic cell.

Chemokines and their receptors constitute an important component in the regulation of directed leukocyte migration. Chemokines are produced at sites of inflammation and attract various leukocytes bearing the corresponding receptors. While the spectrum of chemokines expressed at the inflammatory site can differentially attract 15 certain inflammatory cells, selectivity and variation in chemokine receptor expression on leukocytes provides further regulation to ensure appropriate cell recruitment in response to particular inflammatory stimuli. As the number of identified and characterized chemokine receptors continues to grow, it is becoming increasingly clear that cells selectively express several receptors which may identify, mark, or otherwise 20 characterize functional subsets of leukocytes such as T_H1 and T_H2 , naive and memory, activated and quiescent T cells. Because several characterized and/or orphan chemokine receptors can be co-expressed on individual cells, it has been difficult to validate the role of specific receptors in the initiation and progression of disease or, for that matter, in normal immune function.

25 As described herein, a study of the orphan chemokine receptor GPR-9-6 was conducted. In the course of the study an antibody which binds human GPR-9-6 (mAb 3C3) was produced and used to analyze the expression and function of the receptor on various types of leukocytes. The receptor was found to be expressed predominantly on thymocytes and $\alpha4\beta7^{hi}$ $CD4^+$ memory lymphocytes which home to mucosal sites (e.g.,

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respiratory tract, urogenital tract, alimentary canal and associated tissues (pancreas, gallbladder). As described herein, GPR-9-6 is a functional CC chemokine receptor which binds and is activated by the CC chemokine known as thymus-expressed chemokine (TECK).

- 5 The invention relates to the chemokine receptor GPR-9-6 and to agents (e.g., ligands, antibodies, antagonists, agonists) which bind to the receptor. In one aspect, the invention relates to an antibody which binds to mammalian GPR-9-6 or a portion of GPR-9-6.

Antibodies and Antibody Producing Cells

- 10 The antibody of the invention can be polyclonal or monoclonal, and the term "antibody" is intended to encompass both polyclonal and monoclonal antibodies. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production. The term "antibody" as used herein also encompasses functional fragments of antibodies,
- 15 including fragments of chimeric, humanized, primatized, veneered or single chain antibodies. Functional fragments include antigen-binding fragments which bind to a mammalian GPR-9-6. For example, antibody fragments capable of binding to a mammalian GPR-9-6 or portions thereof, including, but not limited to Fv, Fab, Fab' and F(ab')₂ fragments are encompassed by the invention. Such fragments can be produced
- 20 by enzymatic cleavage or by recombinant techniques. For example, papain or pepsin cleavage can generate Fab or F(ab')₂ fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or F(ab')₂ fragments. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons has been introduced upstream of the natural stop site.
- 25 For example, a chimeric gene encoding a F(ab')₂ heavy chain portion can be designed to include DNA sequences encoding the CH₁ domain and hinge region of the heavy chain.

Single chain antibodies, and chimeric, humanized or primatized (CDR-grafted), or veneered antibodies, as well as chimeric, CDR-grafted or veneered single chain

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antibodies, comprising portions derived from different species, and the like are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, European Patent No. 0 451 216 B1; and Padlan, E.A. *et al.*, EP 0 519 596 A1. See also, Newman, R. *et al.*, *BioTechnology*, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al.*, *Science*, 242: 423-426 (1988) regarding single chain antibodies.

Humanized antibodies can be produced using synthetic or recombinant DNA technology using standard methods or other suitable techniques. Nucleic acid (e.g., cDNA) sequences coding for humanized variable regions can also be constructed using PCR mutagenesis methods to alter DNA sequences encoding a human or humanized chain, such as a DNA template from a previously humanized variable region (see e.g., Kamman, M., *et al.*, *Nucl. Acids Res.*, 17: 5404 (1989)); Sato, K., *et al.*, *Cancer Research*, 53: 851-856 (1993); Daugherty, B.L. *et al.*, *Nucleic Acids Res.*, 19(9): 2471-2476 (1991); and Lewis, A.P. and J.S. Crowe, *Gene*, 101: 297-302 (1991)). Using these or other suitable methods, variants can also be readily produced. In one embodiment, cloned variable regions can be mutated, and sequences encoding variants with the desired specificity can be selected (e.g., from a phage library; see e.g., Krebber *et al.*, U.S. 5,514,548; Hoogenboom *et al.*, WO 93/06213, published April 1, 1993).

Antibodies which are specific for mammalian (e.g., human) GPR-9-6 can be raised against an appropriate immunogen, such as isolated and/or recombinant human GPR-9-6 or portions thereof (including synthetic molecules, such as synthetic peptides).

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Antibodies can also be raised by immunizing a suitable host (e.g., mouse) with cells that express GPR-9-6, such as thymocytes. In addition, cells expressing a recombinant mammalian GPR-9-6 such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor (See e.g., Chuntharapai *et al.*, *J. Immunol.*, 5 152: 1783-1789 (1994); Chuntharapai *et al.*, U.S. Patent No. 5,440,021).

Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed using any suitable technique. A variety of methods have been described (see e.g., Kohler *et al.*, *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein *et al.*, *Nature* 266: 550-552 (1977); Koprowski *et* 10 *al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.*, Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a 15 myeloma cell line such as SP2/0, P3X63Ag8.653 or a heteromyeloma) with antibody producing cells. Antibody producing cells can be obtained from the peripheral blood or, preferably the spleen or lymph nodes, of humans or other suitable animals immunized with the antigen of interest. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce 20 antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating antibodies of the requisite specificity (e.g., human antibodies or antigen-binding fragments) can be used, including, for example, methods which select recombinant antibody from a library (e.g., a phage display library), or which rely upon immunization of transgenic animals (e.g., 25 mice) capable of producing a repertoire of human antibodies (see e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 2551-2555 (1993); Jakobovits *et al.*, *Nature*, 362: 255-258 (1993); Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807; Lonberg *et al.*, WO97/13852).

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In one embodiment, the antibody or antigen-binding fragment thereof has specificity for a mammalian GPR-9-6, preferably a naturally occurring or endogenous human GPR-9-6. In another embodiment, the antibody is an IgG or antigen-binding fragment of an IgG. In another embodiment, the antibody or antigen-binding fragment
5 can bind to a mammalian GPR-9-6 and inhibit (reduce or prevent) one or more functions of the receptor. In a preferred embodiment, the antibody or antigen-binding fragment can inhibit binding of a ligand (i.e., one or more ligands) to the receptor, and/or one or more functions mediated by GPR-9-6 in response to ligand binding.

In a particular embodiment, the antibody or antigen-binding fragment can inhibit
10 the binding of a mammalian (e.g., human) TECK to mammalian (e.g., human) GPR-9-6 and/or one or more functions mediated by GPR-9-6 in response to TECK binding. In a particularly preferred embodiment, the antibody or antigen-binding fragment can inhibit the binding of TECK to GPR-9-6 and, thereby inhibit TECK-induced chemotaxis.

As shown herein, TECK is a ligand for GPR-9-6 and activates the receptor
15 leading to TECK-induced Ca^{2+} flux in cells that express GPR-9-6 (Figure 9A). Cells that express mammalian GPR-9-6, including recombinant cells, can also undergo TECK-induced chemotaxis (Figures 8A-8D, 8F, 10, 11A-11B and 13A-13B). Other functions which can be mediated by GPR-9-6 in response to ligand binding (e.g., TECK) include, for example, signal transduction (e.g., GDP/GTP exchange by GPR-9-6
20 associated G proteins, transient increase in the concentration of cytosolic free calcium [Ca^{2+}]_i) and GPR-9-6-mediated processes and cellular responses (e.g., proliferation, migration, chemotaxis, secretion, degranulation, inflammatory mediator release (such as release of bioactive lipids such as leukotrienes (e.g., leukotriene C_4)), respiratory burst).

In another embodiment, the binding of the antibody or antigen-binding fragment
25 thereof to mammalian (e.g., human) GPR-9-6 can be inhibited by a peptide that consists of the amino acid sequence of SEQ ID NO:3.

As described herein, an antibody designated "mAb 3C3" that binds human GPR-9-6 has been produced. mAb 3C3 can be produced by murine hybridoma 3C3, also referred to as murine hybridoma LS129-3C3-E3-1 which was deposited on March 4,

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1999, on behalf of LeukoSite, Inc., 215 First Street, Cambridge, MA 02142, U.S.A., at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110, U.S.A., under Accession No. _____. In another embodiment, the anti-GPR-9-6 antibody of the invention is mAb 3C3 or an antigen-binding fragment thereof. In another embodiment, the binding of the antibody or antigen-binding fragment to mammalian (e.g., human) GPR-9-6 can be inhibited by mAb 3C3. Such inhibition can be the result of competition for the same or similar epitope, steric interference or due to a change in the conformation of GPR-9-6 that is induced upon antibody binding to the receptor. In still another embodiment, the antibody or antigen-binding fragment of the invention has the same or similar epitopic specificity as mAb 3C3. Antibodies with an epitopic specificity which is the same as or similar to that of mAb 3C3 can be identified by a variety of suitable methods. For example, an antibody with the same or similar epitopic specificity as mAb 3C3 can be identified based upon the ability to compete with mAb 3C3 for binding to mammalian GPR-9-6. In another example, the binding of mAb 3C3 and the binding of an antibody with the same or similar epitopic specificity to mammalian GPR-9-6 can be inhibited by a single peptide (e.g., natural peptide, synthetic peptide). The peptide can comprise nine to about fifty amino acids. Preferably, the peptide comprises nine to about twenty-six amino acids. In still another example, an antibody with the same or similar epitopic specificity as mAb 3C3 can be identified using chimeric receptors (see e.g., Rucker *et al.*, *Cell* 87:437-446 (1996)).

The invention also relates to a bispecific antibody, or functional fragment thereof (e.g., F(ab')₂), which binds to a mammalian GPR-9-6 and least one other antigen. In a particular embodiment, the bispecific antibody, or functional fragment thereof has the same or similar epitopic specificity as mAb 3C3 and least one other antibody (see, e.g., U.S. Patent No. 5,141,736 (Iwasa *et al.*), U.S. Patent Nos. 4,444,878, 5,292,668, 5,523,210 (all to Paulus *et al.*) and U.S. Patent No. 5,496,549 (Yamazaki *et al.*)).

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In a preferred embodiment, the antibody or antigen-binding fragment of the invention specifically binds to a mammalian GPR-9-6. As used herein the term "specific antibody" or "specific" when referring to an antibody-antigen interaction is used to indicate that the antibody can selectively bind to a mammalian GPR-9-6, rather than to indicate that the antibody can bind to only one antigen. For example, an antibody may bind to one or several antigens with low affinity and bind to human GPR-9-6 with a high affinity. Such an antibody is considered to be specific for human GPR-9-6 because when used (e.g., in therapeutic or diagnostic application) at a suitable concentration, the antibody can selectively bind to human GPR-9-6. The concentration of antibody required to provide selectivity for a mammalian GPR-9-6 (e.g., a concentration which reduces or eliminates low affinity binding) can be readily determined by suitable methods, for example, titration.

In another aspect, the invention relates to an isolated cell which produces an antibody or an antigen-binding fragment of an antibody that binds to a mammalian GPR-9-6. In a preferred embodiment, the isolated antibody-producing cell of the invention is an immortalized cell, such as a hybridoma, heterohybridoma, lymphoblastoid cell or a recombinant cell. The antibody-producing cells of the present invention have uses other than for the production of antibodies. For example, the cell of the present invention can be fused with other cells (such as suitably drug-marked human myeloma, mouse myeloma, human-mouse heteromyeloma or human lymphoblastoid cells) to produce, for example, additional hybridomas, and thus provide for the transfer of the genes encoding the antibody. In addition, the cell can be used as a source of nucleic acids encoding the anti-GPR-9-6 immunoglobulin chains, which can be isolated and expressed (e.g., upon transfer to other cells using any suitable technique (see e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Winter, U.S. Patent No. 5,225,539)). For instance, clones comprising a sequence encoding a rearranged anti-GPR-9-6 light and/or heavy chain can be isolated (e.g., by PCR) or cDNA libraries can be prepared from mRNA isolated from the cell lines, and cDNA clones encoding an anti-GPR-9-6 immunoglobulin chain(s) can be isolated. Thus, nucleic acids encoding the heavy

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and/or light chains of the antibodies or portions thereof can be obtained and used for the production of the specific immunoglobulin, immunoglobulin chain, or variants thereof (e.g., humanized immunoglobulins) in a variety of host cells or in an *in vitro* translation system. For example, the nucleic acids, including cDNAs, or derivatives thereof

5 encoding variants such as a humanized immunoglobulin or immunoglobulin chain, can be placed into suitable prokaryotic or eukaryotic vectors (e.g., expression vectors) and introduced into a suitable host cell by an appropriate method (e.g., transformation, transfection, electroporation, infection), such that the nucleic acid is operably linked to one or more expression control elements (e.g., in the vector or integrated into the host

10 cell genome), to produce a recombinant antibody-producing cell.

The antibody of the invention can be produced by any suitable method, for example, by collecting serum from an animal (e.g., mouse, human, transgenic mouse) which has been immunized with a mammalian GPR-9-6. In another example, a suitable antibody producing cell (e.g., hybridoma, heterohybridoma, lymphoblastoid cell,

15 recombinant cell) can be maintained, either *in vitro* or *in vivo*, under conditions suitable for expression (e.g., in the presence of inducer, suitable media supplemented with appropriate salts, growth factors, antibiotic, nutritional supplements), whereby the antibody or antigen-binding fragment is produced. If desired, the antibody or antigen-binding fragment can be recovered and/or isolated (e.g., from the host cells, culture

20 medium) and purified to the desired degree. Recovery and purification of the antibody can be achieved using suitable methods, such as, centrifugation, filtration, column chromatography (e.g., ion-exchange, gel filtration, hydrophobic-interaction, affinity), preparative native electrophoresis, precipitation and ultrafiltration. It will be appreciated that the method of production encompasses expression in a host cell of a transgenic

25 animal (see e.g., WO 92/03918, GenPharm International, published March 19, 1992).

As described herein, antibodies and functional fragments thereof of the present invention can inhibit (reduce or prevent) binding of a ligand to a mammalian GPR-9-6 and/or inhibit one or more functions associated with binding of the ligand to GPR-9-6.

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As discussed below various methods can be used to assess inhibition of binding of a ligand to GPR-9-6 and/or function associated with binding of the ligand to the receptor.

Binding Assays

The invention also relates to methods for detecting or identifying an agent (i.e.,
5 molecule or compound) which can bind to a mammalian GPR-9-6 or a ligand-binding variant thereof.

As used herein "mammalian GPR-9-6" refers to naturally occurring or endogenous mammalian GPR-9-6 proteins and to proteins having an amino acid sequence which is the same as that of a naturally occurring or endogenous
10 corresponding mammalian GPR-9-6 protein (e.g., recombinant proteins, synthetic proteins (i.e., produced using the methods of synthetic organic chemistry)).

Accordingly, as defined herein, the term includes mature receptor protein, polymorphic or allelic variants, and other isoforms of a mammalian GPR-9-6 (e.g., produced by alternative splicing or other cellular processes), and modified or unmodified forms of
15 the foregoing (e.g., lipidated, glycosylated, unglycosylated). Naturally occurring or endogenous mammalian GPR-9-6 proteins include wild type proteins such as mature GPR-9-6, polymorphic or allelic variants and other isoforms which occur naturally in mammals (e.g., humans, non-human primates). Such proteins can be recovered or isolated from a source which naturally produces mammalian GPR-9-6, for example.
20 These proteins and mammalian GPR-9-6 proteins having the same amino acid sequence as a naturally occurring or endogenous corresponding mammalian GPR-9-6, are referred to by the name of the corresponding mammal. For example, where the corresponding mammal is a human, the protein is designated as a human GPR-9-6 protein (e.g., a recombinant human GPR-9-6 produced in a suitable host cell).

25 "Functional variants" of mammalian GPR-9-6 proteins include functional fragments, functional mutant proteins, and/or functional fusion proteins which can be produce using suitable methods (e.g., mutagenesis (e.g., chemical mutagenesis, radiation mutagenesis), recombinant DNA techniques). A "functional variant" is a

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protein or polypeptide which has at least one function characteristic of a mammalian GPR-9-6 protein as described herein, such as a binding activity, a signaling activity and/or ability to stimulate a cellular response. Preferred functional variants can bind a ligand (i.e., one or more ligands, such as TECK).

- 5 Generally, fragments or portions of mammalian GPR-9-6 proteins include those having a deletion (i.e., one or more deletions) of an amino acid (i.e., one or more amino acids) relative to the mature mammalian GPR-9-6 protein (such as N-terminal, C-terminal or internal deletions). Fragments or portions in which only contiguous amino acids have been deleted or in which non-contiguous amino acids have been
10 deleted relative to mature mammalian GPR-9-6 protein are also envisioned.

- Mutant mammalian GPR-9-6 proteins include natural or artificial variants of a mammalian GPR-9-6 protein differing by the addition, deletion and/or substitution of one or more contiguous or non-contiguous amino acid residues (e.g., receptor chimeras). Such mutations can occur at one or more sites on a protein, for example a conserved
15 region or nonconserved region (compared to other chemokine receptors or G-protein coupled receptors), extracellular region, cytoplasmic region, or transmembrane region.

- Fusion proteins encompass polypeptides comprising a mammalian GPR-9-6 (e.g., human GPR-9-6) or a variant thereof as a first moiety, linked via a covalent bond (e.g., a peptide bond) to a second moiety not occurring in the mammalian GPR-9-6 as
20 found in nature. Thus, the second moiety can be an amino acid, oligopeptide or polypeptide. The second moiety can be linked to the first moiety at a suitable position, for example, the N-terminus, the C-terminus or internally. In one embodiment, the fusion protein comprises an affinity ligand (e.g., an enzyme, an antigen, epitope tag, a binding domain) as the first moiety, and a second moiety comprising a linker sequence
25 and human GPR-9-6 or a portion thereof. Additional (e.g., third, fourth) moieties can be present as appropriate.

 In one embodiment, a functional variant of mammalian GPR-9-6 (e.g., a ligand binding variant) shares at least about 80% amino acid sequence similarity with said mammalian GPR-9-6, preferably at least about 90% amino acid sequence similarity, and

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more preferably at least about 95% amino acid sequence similarity with said mammalian GPR-9-6. In another embodiment, a functional fusion protein comprises a first moiety which shares at least about 85% sequence similarity with a mammalian GPR-9-6, preferably at least about 90% sequence similarity, and more preferably at least about 95% sequence similarity with a mammalian GPR-9-6 (e.g., a human GPR9-6 (e.g., SEQ ID NO:2)). In another embodiment, a functional mammalian GPR-9-6 protein or functional variant of a mammalian GPR-9-6 protein shares at least about 80% amino acid sequence similarity, preferably at least about 90% amino acid sequence similarity, and more preferably at least about 95% amino acid sequence similarity with a naturally occurring human GPR-9-6 (e.g., SEQ ID NO:2). Amino acid sequence similarity can be determined using a suitable sequence alignment algorithm, such as the Lasergene system (DNASTAR, Inc., Madison, WI), using the Clustal method with the PAM 250 residue weight table, a gap penalty of 10, a gap length penalty of 10 and default parameters (pairwise alignment parameters: ktuple = 1, gap penalty = 3, window = 4 and diagonals saved = 5). In another embodiment, a functional variant is encoded by a nucleic acid sequence which is different from the naturally-occurring nucleic acid sequence, but which, due to the degeneracy of the genetic code, encodes mammalian GPR-9-6 or a portion thereof.

A composition comprising a mammalian GPR-9-6 or functional variant thereof can be used in a binding assay to detect and/or identify agents that can bind to the receptor. Compositions suitable for use in a binding assay include, for example, cells which naturally express a mammalian GPR-9-6 or functional variant thereof (e.g., thymocytes, GPR-9-6⁺ CLA^{-ve} $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ memory lymphocytes, cell lines (e.g., MOLT-4 (ATCC Accession No. CRL-1582), MOLT-13 (M. Brenner, Brigham and Womans Hospital, Boston, MA)) and recombinant cells comprising an exogenous nucleic acid sequence which encodes a mammalian GPR-9-6 or functional variant thereof. Compositions suitable for use in a binding assay also include, membrane preparations which comprise a mammalian GPR-9-6 or functional variant thereof. Such membrane preparations can contain natural (e.g., plasma membrane) or synthetic

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membranes. Preferably, the membrane preparation is a membrane fraction of a cell that expresses a mammalian GPR-9-6 or a functional variant thereof.

In one embodiment, the method of detecting or identifying an agent that binds to a mammalian GPR-9-6 is a competitive binding assay in which the ability of a test agent to inhibit the binding of a reference agent (e.g., a ligand, an antibody) is assessed. For example, the reference agent can be labeled with a suitable label as described herein, and the amount of labeled reference agent required to saturate the GPR-9-6 present in the assay can be determined. A saturating amount of labeled reference agent and various amounts of a test agent can be contacted with a composition comprising a mammalian GPR-9-6 or functional variant thereof under conditions suitable for binding and complex formation determined.

The formation of a complex between the reference agent and the GPR-9-6 or functional variant thereof can be detected or measured directly or indirectly using suitable methods. For example, the agent can be labeled with a suitable label and the formation of a complex can be determined by detection of the label. The specificity of the complex can be determined using a suitable control such as unlabeled agent or label alone. Labels suitable for use in detection of a complex between an agent and a mammalian GPR-9-6 or functional variant thereof include, for example, a radioisotope, an epitope, an affinity label (e.g., biotin, avidin), a spin label, an enzyme, a fluorescent group or a chemiluminescent group.

The capacity of the test agent to inhibit the formation of a complex between the reference agent and a mammalian GPR-9-6 can be reported as the concentration of test agent required for 50% inhibition (IC_{50} values) of specific binding of labeled reference agent. Specific binding is preferably defined as the total binding (e.g., total label in complex) minus the non-specific binding. Non-specific binding is preferably defined as the amount of label still detected in complexes formed in the presence of excess unlabeled reference agent. Reference agents which are suitable for use in the method include molecules and compounds which specifically bind to a mammalian GPR-9-6 or a functional variant thereof, for example, a ligand of GPR-9-6 or an antibody. In a

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preferred embodiment, the reference agent is mAb 3C3. In a particularly preferred embodiment, the reference agent is a mammalian (e.g., human) TECK.

The invention also relates to a method of identifying or isolating an agent (i.e., molecule or compound) which can be used in therapy, as described herein. In one
5 embodiment, the agent is identified or isolated in a competitive binding assay as described above. In another embodiment, cells which express a mammalian GPR-9-6 or a functional variant thereof are maintained under conditions appropriate for expression of receptor. The cells are contacted with an agent (e.g., ligand, antagonist, agonist) under conditions suitable for binding (e.g., in a suitable binding buffer), and the
10 formation of a complex between the agent and a mammalian GPR-9-6 is detected or measured using suitable techniques. For example, the agent can be labeled as described herein and the amount of label present in an agent-GPR-9-6 complex can be determined. The extent of complex formation can be determined relative to a suitable control (e.g., compared with background determined in the absence of agent, compared with binding
15 of a second agent (i.e., a standard, an isotype control), compared with binding of agent to cells that do not express GPR-9-6).

Thus, the invention relates to a method of identifying or isolating an agent for use in treating a subject having an inflammatory disease. In particular embodiments, the method is a method of identifying or isolating an agent for use in treating a subject
20 having an inflammatory disease associated with mucosal tissue, such as Crohn's disease or colitis. In another embodiment, the method is a method of identifying or isolating an agent for use in inhibiting GPR-9-6-mediated homing of leukocytes in a subject. In another embodiment, the method is a method of identifying or isolating an agent for use in modulating a GPR-9-6 function in a subject.

25 According to the method of the present invention, agents can be individually screened or one or more agents can be tested simultaneously according to the methods herein. Where a mixture of compounds is tested, the compounds selected by the processes described can be separated (as appropriate) and identified by suitable methods (e.g., sequencing, chromatography). The presence of one or more compounds (e.g., a

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ligand, inhibitor, promoter) in a test sample can also be determined according to these methods.

Agents which bind to a mammalian GPR-9-6 and which are useful in the therapeutic methods described herein can be identified, for example, by screening libraries or collections of molecules, such as, the Chemical Repository of the National Cancer Institute, in assays described herein or using other suitable methods. Large combinatorial libraries of compounds (e.g., organic compounds, recombinant or synthetic peptides, "peptoids", nucleic acids) produced by combinatorial chemical synthesis or other methods can be tested (see e.g., Zuckerman, R.N. *et al.*, *J. Med. Chem.*, 37: 2678-2685 (1994) and references cited therein; see also, Ohlmeyer, M.H.J. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10922-10926 (1993) and DeWitt, S.H. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6909-6913 (1993), relating to tagged compounds; Rutter, W.J. *et al.* U.S. Patent No. 5,010,175; Huebner, V.D. *et al.*, U.S. Patent No. 5,182,366; and Geysen, H.M., U.S. Patent No. 4,833,092). Where compounds selected from a combinatorial library by the present method carry unique tags, identification of individual compounds by chromatographic methods is possible. In one embodiment, the collection of agents tested according to the method of the invention does not comprise chemokines or mutants or analogues thereof.

Functional Assays

An agent which binds a mammalian GPR-9-6 or a functional variant thereof can be further studied in one or more suitable assays to determine if said agent can modulate (inhibit (reduce or prevent) or promote) one or more functions of GPR-9-6 as described herein. For example, an agent can be tested in an extracellular acidification assay, calcium flux assay, ligand binding assay, chemotaxis assay or assay which monitors degranulation or inflammatory mediator release (see, for example, Hesselgesser *et al.*, *J. Biol. Chem.* 273(25):15687-15692 (1998) and WO 98/02151).

For example, an agent which binds to a mammalian GPR-9-6 can be tested in a leukocyte chemotaxis assay using suitable cells. Suitable cells include, for example,

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cell lines, recombinant cells or isolated cells which express a mammalian GPR-9-6 and undergo GPR-9-6 ligand-induced (e.g., TECK) chemotaxis. In one example, GPR-9-6-expressing recombinant L1.2 cells (see Campbell, *et al. J Cell Biol*, 134:255-266 (1996) regarding L1.2 cells), can be used in a modification of a transendothelial migration assay (Carr, M.W., *et al. T.A., Proc. Natl Acad Sci, USA*, (91):3652 (1994)). The endothelial cells used in this assay are preferably the endothelial cell line, ECV 304, which can be obtained from the American Type Culture Collection (Manassas, VA). Endothelial cells can be cultured on 6.5 mm diameter Transwell culture inserts (Costar Corp., Cambridge, MA) with 3.0 μ m pore size. Culture media for the ECV 304 cells can consist of M199+10% FCS, L-glutamine, and antibiotics. The assay media can consist of equal parts RPMI 1640 and M199 with 0.5% BSA. Two hours before the assay, 2×10^5 ECV 304 cells can be plated onto each insert of the 24 well Transwell chemotaxis plate and incubated at 37°C. Chemotactic factor such as TECK (Peprotech, Rocky Hill, NJ) (diluted in assay medium) can be added to the 24-well tissue culture plates in a final volume of 600 μ L. Endothelial-coated Transwells can be inserted into each well and 10^6 cells of the leukocyte type being studied are added to the top chamber in a final volume of 100 μ L of assay medium. The plate can then be incubated at 37°C in 5% CO₂/95% air for 1-2 hours. The cells that migrate to the bottom chamber during incubation can be counted, for example using flow cytometry. To count cells by flow cytometry, 500 μ L of the cell suspension from the lower chamber can be placed in a tube and relative counts can obtained for a set period of time, for example, 30 seconds. This counting method is highly reproducible and allows gating on the leukocytes and the exclusion of debris or other cell types from the analysis. Alternatively, cells can be counted with a microscope. Assays to evaluate agents that can inhibit or promote chemotaxis can be performed in the same way as control experiment described above, except that agent solutions, in assay media containing up to 1% of DMSO co-solvent, can be added to both the top and bottom chambers prior to addition of the cells. The capacity of an agent to inhibit or promote chemotaxis can be determined by comparing the number of cell that migrate to the bottom chamber in wells which contain the agent, to the number

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of cells which migrate to the bottom chamber in control wells. Control wells can contain equivalent amounts of DMSO, but no agent.

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An agent which binds to a mammalian GPR-9-6 can also be assessed by monitoring cellular responses induced by active receptor, using suitable cells which express a mammalian GPR-9-6 or a functional variant thereof. For instance, exocytosis (e.g., degranulation of cells leading to release of one or more enzymes or other granule components, such as esterases (e.g., serine esterases), perforin, and/or granzymes), inflammatory mediator release (such as release of bioactive lipids such as leukotrienes (e.g., leukotriene C₄)), and respiratory burst, can be monitored by methods known in the art or other suitable methods (see e.g., Taub, D.D. *et al.*, *J. Immunol.*, 155: 3877-3888 (1995), regarding assays for release of granule-derived serine esterases; Loetscher *et al.*, *J. Immunol.*, 156: 322-327 (1996), regarding assays for enzyme and granzyme release; Rot, A. *et al.*, *J. Exp. Med.*, 176: 1489-1495 (1992) regarding respiratory burst; Bischoff, S.C. *et al.*, *Eur. J. Immunol.*, 23: 761-767 (1993) and Baggiolini, M. and C.A. Dahinden, *Immunology Today*, 15: 127-133 (1994)).

In one embodiment, an agent that can inhibit or promote a function of GPR-9-6 is identified by monitoring the release of an enzyme upon degranulation or exocytosis by a cell capable of this function. Cells expressing a mammalian GPR-9-6 or a functional variant thereof can be maintained in a suitable medium under suitable conditions, and degranulation can be induced. The cells are contacted with an agent to be tested, and enzyme release can be assessed. The release of an enzyme into the medium can be detected or measured using a suitable assay, such as an immunological assay, or biochemical assay for enzyme activity.

The medium can be assayed directly, by introducing components of the assay (e.g., substrate, co-factors, antibody) into the medium (e.g., before, simultaneous with or after the cells and agent are combined). The assay can also be performed on medium which has been separated from the cells or further processed (e.g., fractionated) prior to assay. For example, convenient assays are available for enzymes, such as serine

esterases (see e.g., Taub, D.D. *et al.*, *J. Immunol.*, 155: 3877-3888 (1995) regarding release of granule-derived serine esterases).

In another embodiment, cells expressing a mammalian GPR-9-6 or a functional variant thereof are combined with a ligand of GPR-9-6 (e.g., TECK), an agent to be
5 tested is added before, after or simultaneous therewith, and Ca^{2+} flux is assessed. Inhibition of ligand-induced Ca^{2+} flux is indicative that the agent is an inhibitor or antagonist of mammalian GPR-9-6 function.

Cellular adherence can be monitored by methods known in the art or other suitable methods. Engagement of the chemokine receptors of a lymphocyte can cause
10 integrin activation, and induction of adherence to adhesion molecules expressed in vasculature or the perivascular space. In one embodiment, a ligand, inhibitor and/or promoter of GPR-9-6 function is identified by monitoring cellular adherence by a cell capable of adhesion. For example, an agent to be tested can be combined with (a) cells expressing a mammalian GPR-9-6 or a functional variant thereof (preferably non-
15 adherent cells which when transfected with receptor acquire adhesive ability), (b) a composition comprising a suitable adhesion molecule (e.g., a substrate such as a culture well coated with an adhesion molecule, such as fibronectin), and (c) a ligand or promoter (e.g., agonist), and maintained under conditions suitable for ligand- or promoter-induced adhesion. Labeling of cells with a fluorescent dye provides a
20 convenient means of detecting adherent cells. Nonadherent cells can be removed (e.g., by washing) and the number of adherent cells determined. The effect of the agent in inhibiting or enhancing ligand- or promoter-induced adhesion can be indicative of inhibitor or promoter activity, respectively. Agents active in the assay include inhibitors and promoters of binding, signaling, and/or cellular responses. In another
25 embodiment, an agent to be tested can be combined with cells expressing a mammalian GPR-9-6 and a composition comprising a suitable adhesion molecule under conditions suitable for ligand- or promoter-induced adhesion, and adhesion is monitored. Increased adhesion relative to a suitable control is indicative of the presence of a ligand and/or promoter.

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The binding assays and functional assays described above can be used, alone or in combination with each other or other suitable methods, to detect or identify agents which bind a mammalian GPR-9-6 protein and/or modulators (inhibitors, promoters) of a GPR-9-6 protein function. The *in vitro* methods of the present invention can be adapted for high-throughput screening in which large numbers of samples are processed (e.g., a 96-well format). Cells expressing a mammalian GPR-9-6 (e.g., human GPR-9-6) or a functional variant thereof at levels suitable for high-throughput screening can be used, and thus, are particularly valuable in the identification and/or isolation of agents which bind GPR-9-6, and modulators of GPR-9-6 function. Expression of GPR-9-6 can be monitored in a variety of ways. For instance, expression can be monitored using antibodies of the present invention which bind receptor or a portion thereof. Also, commercially available antibodies can be used to detect expression of an antigen- or epitope-tagged fusion protein comprising a receptor protein or polypeptide (e.g., FLAG tagged receptors), and cells expressing the GPR-9-6 at the desired level can be selected (e.g., by flow cytometry).

Models of Inflammation

In vivo models of inflammation are available which can be used to assess the efficacy antibodies and antigen-binding fragments of the invention as well as agents identified by the methods described herein as *in vivo* as therapeutics. For example, leukocyte infiltration upon intradermal injection of a chemokine and an antibody or fragment thereof reactive with mammalian GPR-9-6 into a suitable animal, such as rabbit, mouse, rat, guinea pig or primate (e.g., rhesus macaque) can be monitored (see e.g., Van Damme, J. *et al.*, *J. Exp. Med.*, 176: 59-65 (1992); Zachariae, C.O.C. *et al.*, *J. Exp. Med.* 171: 2177-2182 (1990); Jose, P.J. *et al.*, *J. Exp. Med.* 179: 881-887 (1994)). In one embodiment, skin biopsies are assessed histologically for infiltration of leukocytes (e.g., GPR-9-6⁺ T cells). In another embodiment, labeled cells (e.g., stably transfected cells expressing a mammalian GPR-9-6, labeled with ¹¹¹In for example) capable of chemotaxis and extravasation are administered to the animal. For example,

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an antibody or agent to be assessed which binds a mammalian GPR-9-6 can be administered, either before, simultaneously with or after a GPR-9-6 ligand or agonist (e.g., TECK) is administered to the test animal. A decrease of the extent of infiltration in the presence of antibody or agent as compared with the extent of infiltration in the absence of said antibody or agent is indicative of inhibition.

As described herein, GPR-9-6 is selectively expressed on memory lymphocytes which home to mucosal sites (e.g., CLA^{ve} $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ lymphocytes). Thus, animal models of inflammatory diseases of the mucosa (e.g., respiratory tract, urogenital tract, alimentary canal and associated organs and tissues (e.g., pancreas, liver, gall bladder)) can be used to assess the therapeutic efficacy of GPR-9-6 modulating agents. For example, the antibodies and antigen binding fragments of the invention as well as agents identified by the methods described herein can be studied in the cotton-top tamarin model of inflammatory bowel disease (Podolsky, D.K., *et al.*, *J. Clin. Invest.* 92:372-380 (1993)). The CD45RB^{hi}/SCID model provides a mouse model with similarity to both Crohn's disease and ulcerative colitis (Powrie, F. *et al.*, *Immunity*, 1: 553-562 (1994)). Therapeutic efficacy in this model can be assessed, for example, by using parameters such as inhibition of recruitment of ¹¹¹In-labeled cells to the colon and reduction in the number of CD4⁺ T lymphocytes in the lamina propria of the large intestine after administration (e.g., intravenous (i.v.), intraperitoneally (i.p.) and per oral (p.o.)) of an agent. Knockout mice which develop intestinal lesions similar to those of human inflammatory bowel disease have also been described (Strober, W. and Ehrhardt, R.O., *Cell*, 75: 203-205 (1993)), and NOD mice provide an animal model of insulin-dependent diabetes mellitus.

Diagnostic Applications

The antibodies of the present invention have application in procedures in which GPR-9-6 can be detected on the surface of cells. The receptor provides a marker of the leukocyte cell types in which it is expressed. For example, antibodies raised against a mammalian GPR-9-6 protein or peptide, such as the antibodies described herein (e.g.,

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mAb 3C3), can be used to detect and/or quantify cells expressing a mammalian GPR-9-6. In one embodiment, the antibodies can be used to sort cells which express GPR-9-6 from among a mixture of cells (e.g., to isolate leukocytes which home to the mucosa, such as GPR-9-6⁺ CLA^{-ve} $\alpha 4\beta 7^{+ve}$ CD4⁺ memory T cells). Suitable methods for counting and/or sorting cells can be used for this purpose (e.g., flow cytometry, fluorescence activated cell sorting). Cell counts can be used in the diagnosis of diseases or conditions in which an increase or decrease in leukocyte cell types (e.g., leukocytes which home to the mucosa) is observed.

Furthermore, the antibodies can be used to detect or measure expression of GPR-9-6. For example, antibodies of the present invention can be used to detect or measure a mammalian GPR-9-6 in a biological sample (e.g., cells, tissues or body fluids from an individual such as blood, serum, leukocytes (e.g., activated T lymphocytes), bronchoalveolar lavage fluid, saliva, bowel fluid, biopsy specimens). For example, a sample (e.g., tissue and/or fluid) can be obtained from an individual and a suitable assay can be used to assess the presence or amount of GPR-9-6 protein. Suitable assays include immunological and immunochemical methods such as flow cytometry (e.g., FACS analysis) and enzyme-linked immunosorbent assays (ELISA), including chemiluminescence assays, radioimmunoassay, immuno-blot (e.g., western blot) and immunohistology. Generally, a sample and antibody of the present invention are combined under conditions suitable for the formation of an antibody-GPR-9-6 complex, and the formation of antibody-receptor complex is assessed (directly or indirectly).

The presence of an increased level of GPR-9-6 reactivity in a sample (e.g., a tissue sample) obtained from an individual can be indicative of inflammation and/or leukocyte (e.g., activated T cell) infiltration and/or accumulation associated with an inflammatory disease or condition, such as an inflammatory bowel disease, allograft rejection, delayed type hypersensitivity reaction, or an infection such as a viral or bacterial infection. The presence of a decreased level of GPR-9-6 reactivity in the circulation (e.g., on the surface of circulating lymphocytes) can also be indicative of leukocyte infiltration and/or accumulation at inflammatory sites. The level of

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expression of a mammalian GPR-9-6 protein or variant can also be used to correlate increased or decreased expression of a mammalian GPR-9-6 protein with a particular disease or condition, and in the diagnosis of a disease or condition in which increased or decreased expression of a mammalian GPR-9-6 protein occurs (e.g., increased or decreased relative to a suitable control, such as the level of expression in a normal individual). Similarly, the course of therapy can be monitored by assessing GPR-9-6 immunoreactivity in a sample from a subject. For example, antibodies of the present invention can be used to monitor the number of cells expressing GPR-9-6 in a sample (e.g., blood, tissue) from a subject being treated with an anti-inflammatory or immunosuppressive agent.

Kits for use in detecting the presence of a mammalian GPR-9-6 protein in a biological sample can also be prepared. Such kits can include an antibody or functional fragment thereof which binds to a mammalian GPR-9-6 receptor or portion of said receptor, as well as one or more ancillary reagents suitable for detecting the presence of a complex between the antibody or fragment and GPR-9-6 or portion thereof. The antibody compositions of the present invention can be provided in lyophilized form, either alone or in combination with additional antibodies specific for other epitopes. The antibodies, which can be labeled or unlabeled, can be included in the kits with adjunct ingredients (e.g., buffers, such as Tris, phosphate and carbonate, stabilizers, excipients, biocides and/or inert proteins, e.g., bovine serum albumin). For example, the antibodies can be provided as a lyophilized mixture with the adjunct ingredients, or the adjunct ingredients can be separately provided for combination by the user. Generally these adjunct materials will be present in less than about 5% by weight based on the amount of active antibody, and usually will be present in a total amount of at least about 0.001% by weight based on antibody concentration. Where a second antibody capable of binding to the anti-GPR-9-6 antibody is employed, such antibody can be provided in the kit, for instance in a separate vial or container. The second antibody, if present, is typically labeled, and can be formulated in an analogous manner with the antibody formulations described above. The components (e.g., anti-GPR-9-6

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antibody or antigen-binding fragment thereof, ancillary reagent) of the kit can be packaged separately or together within suitable containment means (e.g., bottle, box, envelope, tube). When the kit comprises a plurality of individually packaged components, the individual packages can be contained within a single larger
5 containment means (e.g., bottle, box, envelope, tube).

Similarly, the present invention also relates to a method of detecting and/or quantifying expression of a mammalian GPR-9-6 receptor or a portion of the receptor by a cell, in which a composition comprising a cell or fraction thereof (e.g., membrane fraction) is contacted with an antibody or functional fragment thereof (e.g., mAb 3C3)
10 which binds to a mammalian GPR-9-6 or portion of the receptor under conditions appropriate for binding of the antibody or fragment thereto, and binding is monitored. Detection of the antibody, indicative of the formation of a complex between antibody and a mammalian GPR-9-6 or a portion thereof, indicates the presence of the receptor. Binding of antibody to the cell can be determined using any suitable method. The
15 method can be used to detect expression of GPR-9-6 on cells from a subject (e.g., in a sample, such as a body fluid, such as blood, saliva or other suitable sample). The level of expression of GPR-9-6 on the surface of cells (e.g., leukocytes) can also be determined, for instance, by flow cytometry, and the level of expression (e.g., staining intensity) can be correlated with disease susceptibility, progression or risk.

20 Methods of Therapy

Modulation of mammalian GPR-9-6 function according to the present invention, through the inhibition or promotion of at least one function characteristic of a mammalian GPR-9-6 protein, provides an effective and selective way of inhibiting or promoting receptor-mediated functions. Once lymphocytes are recruited to a site, other
25 leukocyte types, such as monocytes, may be recruited by secondary signals. Thus, agents which can modulate GPR-9-6 function, including ligands, inhibitors and/or promoters, such as those identified as described herein, can be used to modulate

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leukocyte function (e.g., leukocyte infiltration including recruitment and/or accumulation).

In one aspect, the present invention provides a method of modulating (inhibiting or promoting) an inflammatory response in a subject in need of such therapy, comprising administering an effective amount of an agent which inhibits or promotes mammalian GPR-9-6 function to an individual in need of such therapy. In one embodiment, an effective amount of an agent which inhibits one or more functions of a mammalian GPR-9-6 protein (e.g., a human GPR-9-6) is administered to a subject to inhibit (i.e., reduce or prevent) inflammation. For example, antibodies of the present invention, including mAb 3C3 can be used in the method. As a result, one or more inflammatory processes, such as leukocyte emigration, chemotaxis, exocytosis (e.g., of enzymes) or inflammatory mediator release, is inhibited. For example, leukocytic infiltration of inflammatory sites (e.g., in a inflamed mucus membrane (e.g., colon, small intestine)) can be inhibited according to the present method. In another embodiment, an effective amount of an agent which inhibits one or more functions of a mammalian GPR-9-6 protein (e.g., a human GPR-9-6) is administered to a subject to inhibit (i.e., reduce or prevent) GPR-9-6-mediated homing of leukocytes.

In another embodiment, an agent (e.g., receptor agonist) which promotes one or more functions of a mammalian GPR-9-6 protein (e.g., a human GPR-9-6) is administered to induce (trigger or enhance) the recruitment of cells to a desired site or to induce an inflammatory response, such as leukocyte emigration, chemotaxis, exocytosis (e.g., of enzymes) or inflammatory mediator release, resulting in the beneficial stimulation of inflammatory processes. For example, T cells can be recruited to combat viral, bacterial or fungal infections.

Thus, the invention relates to a method of treating a subject having an inflammatory disease, comprising administering an effective amount of an antagonist of GPR-9-6 function. In a particular embodiment, the subject has an inflammatory bowel disease, such as Crohn's disease or colitis.

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The invention also relates to a method of inhibiting GPR-9-6-mediated homing of leukocytes in a subject, comprising administering an effective amount of an antagonist of GPR-9-6 function, for example, the homing of leukocytes to mucosal sites can be inhibited.

- 5 In a another embodiment, the invention relates to a method of promoting GPR-9-6 mediated homing of leukocytes in a subject, comprising administering an effective amount of a promoter (e.g., agonist) of GPR-9-6 function.

The term "subject" is defined herein to include animals such as mammals, including, but not limited to, primates (e.g., humans), cows, sheep, goats, horses, dogs, cats, rabbits, guinea pigs, rats, mice or other bovine, ovine, equine, canine, feline, rodent
10 or murine species. Diseases and conditions associated with inflammation and/or infection can be treated using the methods described herein. In a preferred embodiment, the disease or condition is one in which the actions of lymphocytes, particularly lymphocytes which home to mucosal tissues, are to be inhibited or promoted for
15 therapeutic (including prophylactic) purposes. In a particularly preferred embodiment, the inflammatory disease or condition is a T cell-mediated disease or condition.

Examples of inflammatory diseases associated with mucosal tissues which can be treated according to the present method include mastitis (mammary gland), vaginitis, cholecystitis, cholangitis or pericholangitis (bile duct and surrounding tissue of the
20 liver), chronic bronchitis, chronic sinusitis, asthma, and graft versus host disease (e.g., in the gastrointestinal tract). As seen in Crohn's disease, inflammation often extends beyond the mucosal surface, accordingly chronic inflammatory diseases of the lung which result in interstitial fibrosis, such as interstitial lung diseases (ILD) (e.g., idiopathic pulmonary fibrosis, or ILD associated with rheumatoid arthritis, or other
25 autoimmune conditions), hypersensitivity pneumonitis, collagen diseases, sarcoidosis, and other idiopathic conditions can be amenable to treatment. Pancreatitis and insulin-dependent diabetes mellitus are other diseases which can be treated using the present method.

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In a particularly preferred embodiment, diseases which can be treated accordingly include inflammatory bowel disease (IBD), such as ulcerative colitis, Crohn's disease, ileitis, Celiac disease, nontropical Sprue, enteritis, enteropathy associated with seronegative arthropathies, microscopic or collagenous colitis, eosinophilic gastroenteritis, or pouchitis resulting after proctocolectomy, and ileoanal anastomosis.

Additional diseases or conditions, including chronic diseases, of humans or other species which can be treated with inhibitors of GPR-9-6 function, include, but are not limited to:

- 10 • inflammatory or allergic diseases and conditions, including systemic anaphylaxis or hypersensitivity responses, drug allergies (e.g., to penicillin, cephalosporins), insect sting allergies; psoriasis and inflammatory dermatoses such as dermatitis, eczema, atopic dermatitis, allergic contact dermatitis, urticaria; vasculitis (e.g., necrotizing, cutaneous, and hypersensitivity vasculitis); spondyloarthropathies;
 - 15 scleroderma; respiratory allergic diseases such as asthma, allergic rhinitis;
- autoimmune diseases, such as arthritis (e.g., rheumatoid arthritis, psoriatic arthritis), multiple sclerosis, systemic lupus erythematosus, myasthenia gravis, juvenile onset diabetes, glomerulonephritis and other nephritides, autoimmune thyroiditis, Behcet's disease;
- 20 • graft rejection (e.g., in transplantation), including allograft rejection or graft-versus-host disease;
- other diseases or conditions in which undesirable inflammatory responses are to be inhibited can be treated, including, but not limited to, atherosclerosis, myositis (including polymyositis, dermatomyositis).

Diseases or conditions of humans or other species which can be treated with promoters (e.g., an agonist) of GPR-9-6 function, include, but are not limited to:

- infectious diseases, such as bacterial infections and tuberculoid leprosy, and especially viral infections;

- 5 • immunosuppression, such as that in individuals with immunodeficiency syndromes such as AIDS, individuals undergoing radiation therapy, chemotherapy, or other therapy which causes immunosuppression; immunosuppression due congenital deficiency in receptor function or other causes.

10 Modes of Administration

According to the method, one or more agents can be administered to the subject by an appropriate route, either alone or in combination with another drug. An effective amount of an agent (e.g., a molecule which inhibits ligand binding, an anti-GPR-9-6 antibody or antigen-binding fragment thereof) is administered. An effective amount is
15 an amount sufficient to achieve the desired therapeutic or prophylactic effect, under the conditions of administration, such as an amount sufficient for inhibition or promotion of GPR-9-6 receptor function, and thereby, inhibition or promotion, respectively, of a GPR-9-6-mediated process (e.g., an inflammatory response). The agents can be administered in a single dose or multiple doses. The dosage can be determined by
20 methods known in the art and is dependent, for example, upon the particular agent chosen, the subject's age, sensitivity and tolerance to drugs, and overall well-being. Suitable dosages for antibodies can be from about 0.01 mg/kg to about 100 mg/kg body weight per treatment.

A variety of routes of administration are possible including, for example, oral,
25 dietary, topical, transdermal, rectal, parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous injection, intradermal injection), and inhalation (e.g., intrabronchial, intranasal or oral inhalation, intranasal drops) routes of administration,

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depending on the agent and disease or condition to be treated. Administration can be local or systemic as indicated. The preferred mode of administration can vary depending upon the particular agent (e.g., GPR-9-6 antagonist) chosen, and the particular condition (e.g., disease) being treated, however, oral or parenteral administration is generally preferred.

The agent can be administered as a neutral compound or as a salt. Salts of compounds containing an amine or other basic group can be obtained, for example, by reacting with a suitable organic or inorganic acid, such as hydrogen chloride, hydrogen bromide, acetic acid, perchloric acid and the like. Compounds with a quaternary ammonium group also contain a counteranion such as chloride, bromide, iodide, acetate, perchlorate and the like. Salts of compounds containing a carboxylic acid or other acidic functional group can be prepared by reacting with a suitable base, for example, a hydroxide base. Salts of acidic functional groups contain a counteranion such as sodium, potassium and the like.

The agent can be administered to the individual as part of a pharmaceutical composition for modulation of GPR-9-6 function comprising an inhibitor or promotor of GPR-9-6 function and a pharmaceutically acceptable carrier. Formulation will vary according to the route of administration selected (e.g., solution, emulsion, capsule). Suitable pharmaceutical carriers can contain inert ingredients which do not interact with the promoter (agonist) or inhibitor (antagonist) of GPR-9-6 function. Standard pharmaceutical formulation techniques can be employed, such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's-lactate and the like. Methods for encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art (Baker, *et al.*, "Controlled Release of Biological Active Agents", John Wiley and Sons, 1986). For inhalation, the agent can be

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solubilized and loaded into a suitable dispenser for administration (e.g., an atomizer, nebulizer or pressurized aerosol dispenser).

Furthermore, where the agent is a protein or peptide, the agent can be administered via *in vivo* expression of the recombinant protein. *In vivo* expression can be accomplished via somatic cell expression according to suitable methods (see, e.g. U.S. Patent No. 5,399,346). In this embodiment, a nucleic acid encoding the protein can be incorporated into a retroviral, adenoviral or other suitable vector (preferably, a replication deficient infectious vector) for delivery, or can be introduced into a transfected or transformed host cell capable of expressing the protein for delivery. In the latter embodiment, the cells can be implanted (alone or in a barrier device), injected or otherwise introduced in an amount effective to express the protein in a therapeutically effective amount.

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

EXEMPLIFICATION

Purification of Cell Populations

Human peripheral blood was collected in 10% (v/v) 0.1 M EDTA, layered onto 1-Step Polymorphs gradient (1.113 ± 0.01 g/ml, Accurate Chemical Co., Westbury, NY) and centrifuged at $400 \times g$ for 30 minutes at room temperature. Neutrophil and mononuclear cell layers were collected, re-suspended in Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (Life Technologies, Grand Island, NY) and centrifuged for 15 minutes at $\sim 750 \times g$. Red blood cells were lysed in the neutrophil fraction by re-suspending the pellet in E-Lyse (5 ml/ 10^7 cells)(Cardinal Associates, Santa Fe, NM) for 5 minutes on ice. Both cell fractions were washed 2 times with ice cold DPBS. The mononuclear cells were allowed to adhere to protein coated plastic for 2-3 hours and then non-adherent cells were gently washed off the plate. After a further 12 hours the non-adherent dendritic cells were washed off the plate and depleted of B lymphocytes and T lymphocytes with anti-CD19 and anti-CD2 coated magnetic beads

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(Dynabeads; Dynal, Oslo, Norway) (5 beads per cell). The remaining cells were cultured in 50 ng/ml granulocyte macrophage colony stimulating factor (GM-CSF, R and D Systems, Minneapolis, MN) and 40 ng/ml IL-4 (R and D Systems) Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) 10% fetal calf serum (FCS, HyClone, Logan, UT) plus additives: penicillin 50U/ml, streptomycin 50 µg/ml, L-glutamine 2mM, HEPES 10mM, MEM sodium pyruvate 10 mM, MEM nonessential amino acids 0.1 mM and 2-mercaptoethanol 5.5×10^{-5} M (all from Gibco BRL, Grand Island, NY) for 7 days (Sallusto, F. and Lanzavecchia, A., *J. Exp. Med.*, 179:1109-1118 (1994)) to generate immature dendritic cells (IMDC) and in some cases 24 hours further culture in 10 ng/ml LPS was used to mature the dendritic cells. CD4⁺, CD8⁺, CD14⁺, CD56⁺ and CD19⁺ populations were purified from mononuclear cells with the relevant Miltenyi Beads (Miltenyi Biotek, Bergisch Gladbach, Germany) using 20 µl of beads for 10^7 mononuclear cells in PBS/1% BSA/5 mM EDTA at 5×10^7 cells/ml for 30 minutes at 4°C. They were then spun down, re-suspended in PBS/1% BSA/5 mM EDTA and 5×10^7 cells/ml and passed over a VS column (Miltenyi Biotech, Auburn, CA 95603) in a magnetic field to remove non-tagged cells. Cells were removed by forcing 20 ml of PBS/1% BSA/5 mM EDTA over the VS column, outside the magnetic field.

Antibodies and Reagents

Labeled antibodies which bind to: CD4, CD8, CD14, CD19, CD49d, CD56, CD62L, CLA, CD45RA, CD45RO, CXCR5, CD80 and CD86 were obtained from Pharmingen (San Diego, CA) and used for immunofluorescence studies, while anti-αE and Anti-CD83 were obtained from Beckman Coulter (Fullerton, CA). OKT3, a anti-human CD3 mAb, was obtained from American Type Culture Collection (ATCC, Manassas, VA) and anti-human CD28 mAb was obtained from Becton Dickinson (Mountain View, CA). Some of the anti-chemokine receptor mAbs were produced at LeukoSite, Inc. (Cambridge, MA) and have the clone names anti-CCR3 (7B11), anti-CCR4 (2B10), anti-CCR6 (11A9) and anti-CXCR3 (1C6). Several anti-chemokine

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receptor mAbs used in FACS analysis were obtained from commercial sources. Anti-CCR2, anti-CCR6 and anti-CXCR5 mAbs used for immunofluorescence studies were obtained from R and D Systems (Minneapolis, MN), while anti-CCR5 and anti-CXCR4 were obtained from Pharmingen (San Diego, CA). Recombinant human chemokines were obtained from Peprotech (Rocky Hill, NJ) and R&D Systems (Minneapolis, MN) and in some cases synthesized using solid phase methods that were optimized and adapted to a fully automated peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA) as described (Clark-Lewis, I., *et al.*, *Biochemistry*, 30:3128-3135 (1991)). The human endothelial cell line ECV304 was purchased from ATCC. All cytokines were obtained from R&D Systems (Minneapolis, MN).

Generation of Anti-GPR-9-6 mAbs

A peptide consisting of the NH₂ terminus of GPR-9-6 was generated having the sequence MADDYGSESTSSMEDYVNFNFTDFYC (SEQ ID NO:3). BALB/C mice were immunized i.p. with 10 µg of GPR-9-6 peptide/KLH conjugate prepared in Freund's Complete Adjuvant (FCA, Sigma, St. Louis, MO) at day 1, 10 µg of GPR-9-6 peptide/KLH conjugate prepared in Incomplete Freund's Adjuvant (IFA, Sigma, St. Louis, MO) at day 20, and 10 µg of GPR-9-6 peptide/KLH conjugate prepared in PBS at day 40. At day 60, the mice were boosted with 10 µg of GPR-9-6 peptide/KLH in PBS, and after 4 days, the spleens were removed and fused to SP2/0 myeloma cells (ATCC) (Coligan *et al.*, *Current Protocols in Immunology* 2.5.1 (1992)). Fusions were screened by ELISA, using plates coated with GPR-9-6 peptide. Hybridomas producing anti-GPR-9-6 mAbs were checked for reactivity with GPR-9-6 transfectants and subcloned for further characterization. Murine hybridoma 3C3, also referred to as hybridoma LS129-3C3-E3-1, can be cultivated at 37°C in an 5% CO₂ atmosphere in DMEM supplemented with FCS (10%), IL-6 (100 ng/ml), penicillin (50U/ml), streptomycin (50 µg/ml), L-glutamine (2 mM), HEPES (10 mM), MEM sodium pyruvate (10 mM), MEM nonessential amino acids (0.1 mM) and 2-mercaptoethanol (5.5 x 10⁻⁵M).

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Preparation of Chronically Activated T_H1 and T_H2 Lymphocytes

- As previously described (Murphy, E., *et al.*, *J. Exp. Med.*, 183:901-913 (1997)), six-well Falcon plates were coated overnight with 10 μ g/ml anti-CD28 and 2 μ g/ml OKT3, and then washed twice with PBS. Umbilical cord blood CD4⁺ lymphocytes (Poietic Systems, German Town, MD) were cultured at 10⁵ -10⁶ cells/ml in DMEM with 10% FCS and IL-2 (4 ng/ml). IL-12 (5 ng/ml) and anti-IL-4 (1 μ g/ml) were used to direct to T_H1, while IL-4 (5 ng/ml) and anti-IFN gamma (1 μ g/ml) were used to direct to T_H2. After 4-5 days, the activated T_H1 and T_H2 lymphocytes were washed once in DMEM and cultured for 4-7 days in DMEM with 10% FCS and IL-2 (1 ng/ml).
- Following this, the activated T_H1 and T_H2 lymphocytes were re-stimulated for 5 days with anti-CD28/OKT3 and cytokines as described above, but with the addition of anti-CD95L (1 μ g/ml) to prevent apoptosis. After 4-5 days the T_H1 and T_H2 lymphocytes were washed and then cultured again with IL-2 for 4 days. Activated T_H1 and T_H2 lymphocytes were maintained in this way for a maximum of three cycles.

ECV304 Transmigration and Chemotaxis Assays

- 3 micrometer pore diameter Transwell tissue culture inserts were either used uncoated or coated with 2% gelatin for 2 hours. Then 0.45 ml of DMEM with 5% FCS was placed in the lower wells of the chambers and 2 x 10⁵ EVC304 cells were added to each gelatin coated insert in 0.2 ml of DMEM 5% FCS. After two days, the wells and inserts were washed twice with RPMI-1640 (Gibco BRL, Grand Island, NY) containing 0.5% HSA (human serum albumin), 10 mM HEPES and then chemokine was added to the lower well. The cells under study were washed once in RPMI and re-suspended at 4 x 10⁶ cells/ml for T_H1/T_H2 lymphocytes, cell lines and transfectants, or at 10⁷ cells/ml for resting CD4 lymphocytes in RPMI 0.5% HSA and 10 mM HEPES. An aliquot of 200 μ l of cell suspension (input of 8 x 10⁵ cells and 2 x 10⁶ cells, respectively) was added to each insert. After 2 to 4 hours the inserts were removed and the number of cells which had migrated through the ECV304 monolayer to the lower well counted for 30 seconds on a Becton Dickinson FACScan with the gates set to acquire the cells of

interest. Using this technique, 100% migration would be 25,000 cells for T_H1/T_H2 cells and 75,000 cells for resting CD4 lymphocytes, where this number represents the cells in the lower well counted on the FACScan over 1 minute. To study the phenotype of migrating cells, identical experiments with CD4 lymphocytes were performed with 6
5 well plates using 24 mm diameter inserts. Chemotaxis assays were identical to ECV304 migration assays but Fibronectin coated inserts (10 μ g/ml) were used. In all cases, the data points were the result of duplicate wells, with the mean value shown and the error bars representing the sample standard deviation.

Ca²⁺ Mobilization (Ca²⁺ Flux) Assay

10 10^7 cells/ml in DPBS were labeled for 30 minutes with Fura 2 dye (Molecular Probes, Eugene, OR) at 2 mM, washed three times in DPBS and resuspended at 10^6 cells/ml in DPBS containing 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES, and 5.5 mM glucose. The cells were then analyzed on a fluorimeter (Hitachi model F2000 fluorescence spectrophotometer, excitation 340 nm, emission 510 nm) using 10% NP-
15 40 and 10 mM EDTA to establish the max and min Ca²⁺ mobilizations.

Recombinant DNA Methods

Plasmid DNA was isolated using QIAGEN-tips as recommended by manufacturer (QIAGEN Inc., Chatsworth, CA). DNA ligations, restriction endonuclease digestions, and gel electrophoresis were performed as described
20 previously (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual 2nd ed.*, Cold Spring Harbor Laboratory Press, (Cold Spring Harbor, NY) (1989)). DNA purification through agarose gel extraction was performed using the QIAEXII Gel Extraction Kit as recommended by the manufacturer (QIAGEN Inc., Chatsworth, CA). Plasmid DNA was introduced into *E. coli* by chemical transformation (GIBCO, Inc.).
25 Enzymes were purchased from New England Biolabs, Inc. (Beverly, MA), GIBCO Bethesda Research Laboratories, Inc. (Gaithersburg, MD), or from Boehringer Mannheim, Inc. (Germany). RNA was isolated from frozen tissues or cells using either

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the standard guanidinium isothiocyanate method (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual 2nd ed.*, Cold Spring Harbor Laboratory Press, (Cold Spring Harbor, NY) (1989)) or the RNeasy kit as recommended (QIAGEN Inc., Chatsworth, CA). DNA sequencing was performed by Sequi-Net (Colorado State University) using the FS DyeDeoxy Terminator cycle sequencing kit and a model 377 DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). Sequences were analyzed using SeqMan (DNASTAR, Inc., Madison, WI).

PCR

Primers were designed for use in the PCR to amplify the complete coding region of GPR-9-6 based on the nucleotide sequence deposited in GenBank (U45982)(SEQ ID NO:1) which is incorporated herein by reference. *Bam*HI and *Xba*I sites were incorporated into primer pair BAZ201

5'..TCGAAGGGATCCCTAACATGGCTGATGACTATGGC..3' (SEQ ID NO:4) and BAZ202

5'..AAGAAGTCTAGAACCCCTCAGAGGGAGAGTGCTCC..3' (SEQ ID NO:5) for directional cloning (bold: coding sequence, italic: enzyme site). 5 μ g of total human genomic DNA (Clontech, Palo Alto, CA) was used as the template in the *Pfu* PCR cycles, with 60 mM Tris-HCL, pH 9.5, 1.5 mM MgCl₂, 100 pmol primers, 200 μ M dNTP, and 5 units *Pfu*I polymerase (Invitrogen, Carlsbad, CA) in a 100 μ l volume. The cycle parameters were an initial melt 95°C, 2 minutes, then 35 cycles: 95°C, 30s; 55°C, 30s; 72°C, 2 minutes 15s, followed by a final extension 72°C, 7 minutes in a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, CN).

Primers were designed to amplify the complete coding region of TECK based on the published nucleotide sequence (accession U86358), which is incorporated herein by reference. *Hind*III and *Xba*I sites were incorporated into primer pair BAZ203

5'..TCGAAGAAGCTTATGAACCTGTGGCTCCTG..3' (SEQ ID NO:6) and BAZ204

5'..AAGAAGTCTAGATCACAGTCCTGAATTAGC..3' (SEQ ID NO:7) for directional cloning (bold: coding sequence, italic: enzyme site). 5 μ g of human thymus

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RNA was reverse transcribed with oligo dT in a 20 μ l volume. The cDNA was mixed with 200 μ M dNTP, 100 pmol primers, 60 mM Tris-HCl, pH 9.5, 1.5 mM MgCl₂, and 10 units AmpliTaq polymerase (Perkin-Elmer Roche Molecular Systems, Branchburg, NJ) in a 50 μ l volume. The cycle parameters were an initial melt 95°C, 2 minutes, then
5 35 cycles: 95°C, 30s; 55°C, 30s; 72°C, 1 minute, followed by a final extension 72°C, 7 minutes. The human thymus was obtained from Children's Hospital (Boston, MA).

Semi-quantitative PCR amplification of TECK using primers BAZ203 (SEQ ID NO:6) and BAZ204 (SEQ ID NO:7), and of GPR-9-6 using primers BAZ201 (SEQ ID NO:4) and BAZ202 (SEQ ID NO:5), was performed using equal amounts of cDNA
10 (500 ng) template from thymus, small intestine, colon, brain, lymph node, and spleen, as well as 500 ng genomic DNA (ClonTech, Palo Alto, CA). The same conditions and PCR profile were used as the AmpliTaq PCR cycle described above, except that 30 cycles were performed. Amplification with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (ClonTech, Palo Alto, CA, catalog number 5840-1) was used to
15 demonstrate equivalency of template.

After agarose gel electrophoresis, the PCR products were visualized in the presence of ethidium bromide with a UV light source. DNA fragments of predicted size (~450 bp for TECK and ~1 kb for GPR-9-6) were isolated and cloned into pBluescript II KS+ (Stratagene, Inc., La Jolla, CA) and pcDNA3 (Stratagene, Inc.), respectively, for
20 sequence analysis and further manipulation.

Expression Vector Construction and Generation of a GPR-9-6-expressing Stable Cell Line

The coding region of GPR-9-6 was amplified by PCR and directionally cloned into the *Bam*HI/*Xba*I sites of pcDNA3 (Invitrogen, San Diego, CA). Transfectants were
25 then generated in the murine pre-B lymphoma cell line L1.2, maintained in RPMI-1640 supplemented with 10% fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 50 units/ml Pen/Strep, 0.55 mM β -mercaptoethanol, 10 mM HEPES, and 1 mM sodium pyruvate (Gibco BRL). 20 μ g of linearized GPR-9-6 in pcDNA3 was used to transfect

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the cell line as follows. L1.2 cells were washed twice in PBS and re-suspended in 0.8 ml of the same. The plasmid DNA was mixed with the cells and incubated for 10 minutes at room temperature, transferred to a 0.4-cm electroporation cuvette, and a single pulse was then applied at 250 V, 960 μ F. The electroporation was followed by a 10-min incubation at room temperature. G418 (Geneticin, Gibco BRL) was added to a final concentration of 0.8 mg/ml 48 hours after transfection and the cells were grown in bulk culture under drug selection 2-3 weeks. The transfectants were then stained by mAbs with reactivity against the GPR-9-6 peptide (see below) and analyzed by FACScan (Becton Dickinson & Co., Mountain View, CA) to confirm surface expression of GPR-9-6 and cloned by limiting dilution. Transfected cells were treated with 5 mM n-butyric acid for 24 hours before experimentation (Palmero, D.P., *et al.*, *J. Biotech.*, 19:35-47 (1991)).

Northern Blot Analysis

Northern blots were either purchased from ClonTech or prepared as follows. Total RNA was separated by electrophoresis on 1.2% formaldehyde agarose gels and transferred to a nylon membranes (Hybond-N+; Amersham Corp., Arlington Heights, IL) by the capillary method as described previously (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual 2nd ed.*, Cold Spring Harbor Laboratory Press, (Cold Spring Harbor, NY) (1989)) and crosslinked using a Stratalinker (Stratagene, Inc.). Hybridizations with radio-labeled probes was with ExpressHyb Solution (Clonetech) using the manufacture's suggested protocol. Length of autoradiography exposure is described in appropriate figure legends. Full length gel purified TECK and GPR-9-6 DNA fragments were used in hybridizations.

Results

A mAb Raised to GPR-9-6; mAb 3C3 Selectively Reacts with GPR-9-6 Transfectants

Due to its close phylogenetic association with other known leukocyte chemokine receptors (Figure 1), we cloned GPR-9-6 by PCR using primers designed from the

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deposited GenBank sequence. GPR-9-6/L1.2 transfectants were prepared and stained with mAbs raised against GPR-9-6 in fusions in which mice had been immunized with the first 26 amino acids of the NH₂ terminus of GPR-9-6 (SEQ ID NO:3) coupled to KLH. The mAb, designated mAb 3C3, reacted with GPR-9-6/L1.2 transfectants but not
 5 with parental L1.2 cells. mAb 3C3 was found to have an IgG_{2b} isotype. In cross-reactivity studies, mAb 3C3 did not cross-react with CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7 or CXCR1, CXCR2, CXCR3 and CXCR4 transfectants. The data for CCR6 are shown here, as it is one of the more closely related chemokine receptors to GPR-9-6 (Figures 2A-2B). Also, the NH₂ terminal peptide of GPR-9-6 (SEQ ID NO:3)
 10 was found to completely block the binding of mAb 3C3 to GPR-9-6 transfectants (data not shown), further validating the specificity of this mAb.

GPR-9-6 is Expressed on all B Lymphocytes, Subsets of CD4 Lymphocytes and a Minor Subset of CD8 Lymphocytes in Peripheral Blood, as well as on Thymocytes

In initial two color studies of peripheral blood, GPR-9-6 was found to be
 15 expressed on a small subset (2-4%) of CD4 lymphocytes as well as on a very small subset of CD8 lymphocytes (Figure 3), while B lymphocytes expressed low and heterogeneous levels of GPR-9-6. Monocytes, basophils, eosinophils, neutrophils and NK cells did not express GPR-9-6 under the conditions used (Figure 3). GPR-9-6 was expressed on a large subset of thymocytes expressing all levels of TcR, although a small
 20 subset of TcR^{high}GPR-9-6^{-ve} thymocytes was evident. In three-color experiments, GPR-9-6 was found on the majority of CD4, CD8 and CD4⁺CD8⁺ thymocytes and on approximately 50% of immature CD4^{-ve}CD8^{-ve} thymocytes (data not shown). No expression of GPR-9-6 was seen on either immature or mature dendritic cells (Figure 4D). However, as expected, immature dendritic cells expressed CCR5, which was
 25 down-regulated on LPS activation, while CD83 and CD86 were up-regulated (Figures 4A-4C). In examining a large panel of cell lines GPR-9-6 was found on several T cell lines (Table 1). Umbilical CD4⁺ lymphocytes did not express GPR-9-6 (Figure 4E) and chronic activation of these cells in the presence of IL-12 or IL-4 to generate T_H1 or T_H2

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lymphocytes failed to induce the expression of GPR-9-6 (Figure 4H). However, as expected, CXCR3 were clearly up-regulated on T_H1 lymphocytes (Figure 4F), while $\alpha 4\beta 7$, an integrin utilized in lymphocyte trafficking to mucosal sites, was up-regulated on both T_H1 and T_H2 lymphocytes (Figure 4G).

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- 5 Expression of GPR-9-6 on CD4 lymphocytes and B lymphocytes was measured over time, and was found to be relatively constant (Figure 5A). However, activation of T lymphocytes with anti-CD3 mAb resulted in transient down-regulation of GPR-9-6 over 2 days, with expression recovering after 10 days of culture in IL-2 (Figure 5B). Chemokine receptors CCR6 and CCR5 shown similar changes in expression upon T
- 10 lymphocyte activation (Figure 5C).

The CD4 Lymphocyte Subset that Express GPR-9-6 are Predominantly of Memory Phenotype and Express High Levels of Mucosal Lymphoid Homing Receptor $\alpha 4\beta 7$ but not Skin Homing Receptor CLA

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- 15 The small subset of CD4 lymphocytes that express GPR-9-6 were examined in more detail by three-color staining (Figure 6). The CD4 lymphocytes that express GPR-9-6 were mainly of memory phenotype, and those cells that expressed the highest levels of GPR-9-6 were all of memory phenotype. Interestingly, memory CLA⁺ CD4 lymphocytes, which traffic to skin, did not express GPR-9-6. In contrast, a subset of memory $\alpha 4\beta 7^{\text{high}}$ CD4 lymphocytes, which traffic to mucosal sites, clearly expressed
- 20 GPR-9-6. The subset of memory CD4 lymphocytes defined by expression of $\alpha E\beta 7$ were also clearly subdivided into GPR-9-6 positive and negative subsets. GPR-9-6^{high} CD4 lymphocytes did not express CD62L, a homing receptor which is involved in trafficking to peripheral lymph nodes, while a small subset of GPR-9-6^{dull} CD62L⁺ lymphocytes was evident.

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- 25 GPR-9-6⁺ CD4 lymphocytes were also examined for co-expression of other chemokine receptors known to be expressed on CD4 lymphocytes (Figure 7). While GPR-9-6 was clearly found on both positive and negative subsets of CCR5, CCR6,

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CXCR3 and CXCR5, CD4 lymphocyte expression of CCR2 and GPR-9-6 was mutually exclusive.

GPR-9-6 Chemokine Receptor Specifically Binds to TECK

Out of all the published chemokines tested, only TECK proved able to induce chemotaxis of GPR-9-6/L1.2 transfectants (Figure 8A). MCP-1-4, MIP-1 α , MIP-1 β , eotaxin-1, eotaxin-2, RANTES, I-309, TARC, MDC, MIP4, SLC, HCC1, fractalkine, lymphotactin, MIG, IP-10, ITAC, ADEC, IL-8, gro- α , gro- β , gro- γ , leukotactin, SDF-1 α , SDF-1 β , MIP3 and MIP4 all proved unable to induce chemotaxis of the GPR-9-6/L1.2 transfectants. TECK induced chemotaxis of L1.2/GPR-9-6 transfectants was inhibited by the mAb 3C3, but not by an anti-CCR3 mAb 7B11 (Figure 8B). TECK did not act on any of the other transfectants tested (CCR1, CCR2, CCR 4, CCR5, CCR6, CCR7 and CXCR1, CXCR2, CXCR3, CXCR4, data not shown). Interestingly, TECK was also found to act on the T cell lines MOLT-4 (Figure 8D) and MOLT-13 (Figure 8F), which express GPR-9-6 (Table 1). TECK was not chemotactic for other cell lines, such as SKW3 (Figure 8E), which do not express GPR-9-6. Using the T cell line MOLT-4, TECK induced chemotaxis was shown to be blocked by pertussis toxin (Figure 8C). Additionally, the anti-GPR-9-6 mAb 3C3 blocked the chemotaxis of the MOLT-13 cells to TECK, but had no effect on SDF1 α induced chemotaxis of these cells (Figure 8F). In calcium mobilization experiments, TECK was also found to induce Ca²⁺ flux in GPR-9-6⁺ cell lines such as MOLT-4 (Figures 9A-9C), while chemokines such as MDC for which these cells do not express the relevant receptor had no effect.

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Table 1 GPR-9-6 Expression by Cell Lines

CELL	GPR-9-6	CXCR4
MOLT-4	+	+
MOLT-13	+	+
CEM	-	+
PEER	-	+
HUT78	-	+
PMI	-	+
SKW.3	-	+
JURKAT	-	+
RAMOS	-	+
RAJI	-	+
JY	-	+
THP-1	-	-
U937	-	+
KG1	-	-
HL-60	-	+/-
K562	-	-
EOL-1	-	+
KU812	-	-

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Leukocyte subsets were also tested (Figure 10) to determine if they chemotaxed to TECK. As observed in the mouse, neutrophils, monocytes, eosinophils, CD8 and NK cells did not chemotax to TECK, but did chemotax to other chemokines. However, TECK was chemotactic for a minor subset of CD4 lymphocytes. As murine TECK induces thymocyte chemotaxis, chemotaxis of human thymocytes to TECK and SDF1 α , both of which mediate thymocyte chemotaxis (data not shown) was examined. Anti-GPR-9-6 mAb 3C3 blocked thymocyte and CD4 lymphocyte chemotaxis to TECK. The anti-GPR-9-6 mAb 3C3 had no effect on TARC-induced chemotaxis of CD4 lymphocytes, indicating that the effect is specific (Figures 11A-11C). These results indicate that GPR-9-6 is the major physiological receptor for TECK.

Tissue Distribution of TECK and GPR-9-6 Transcripts

Due to the expression of GPR-9-6 on mucosal homing lymphocytes, the distribution of TECK and GPR-9-6 transcripts in lymphoid and mucosal tissue was examined (Figures 12A-12B). TECK was selectively expressed in thymus and small intestine (Figure 12A), while GPR-9-6 was expressed at high levels in thymus and weakly in spleen and peripheral blood leukocytes (Figure 12B). While GPR-9-6 transcripts were not detected by Northern blot analysis in small intestine, GPR-9-6 message was detected in small intestine, thymus, lymph node and spleen using the more sensitive technique of RT-PCR (Figure 12C). Messages for both TECK and GPR-9-6 were not detected in brain or colon. In other Northern blots, TECK and GPR-9-6 were not detected in T_H1, T_H2, Tr1 (Groux, *et al.*, *Nature* 389:737-742 (1997)) lymphocytes, LAK cells, monocytes, CD34 derived dendritic cells, monocyte derived dendritic cells, astrocytes, human umbilical vein endothelial cells (HUVEC) and pulmonary vein endothelial cells (PUVEC)(data not shown). Finally, GPR-9-6 transcript was shown to be present only in cell lines which had previously been shown to be GPR-9-6⁺ by staining with mAb 3C3, further validating the specificity of the mAb (Figure 12B).

Only $\alpha 4\beta 7^{\text{high}}$ CD4 and CD8 Lymphocytes Migrate to TECK

As GPR-9-6 is expressed mainly on memory $\alpha 4\beta 7^{\text{high}}$ CD4 lymphocytes, CD45RA^{-ve} memory CD4 and CD8 lymphocytes which expressed none, intermediate or high levels of $\alpha 4\beta 7$ were isolated. Only $\alpha 4\beta 7^{\text{high}}$ memory CD8 lymphocytes and
 5 $\alpha 4\beta 7^{\text{+ve}}$ CLA^{-ve} memory CD4 lymphocytes chemotaxed to TECK (Figures 13A-13B).

Discussion

Several different adhesion molecules are involved in trafficking of lymphocyte subsets to distinct physiologic location, such as peripheral lymph node (Gallatin, W.M., *et al.*, *Nature*, 304:30-34 (1983)), Peyer's Patches (Hamman, A., *et al.*, *J. Immunol.*, 152:3282-3292 (1994); Andrew, D.P., *et al.*, *Eur. J. Immunol.*, 26:897-905 (1996)) and inflammatory sites (Frenette, P.S., *et al.*, *Cell*, 84:563-574 (1996); Tietz, W.Y., *et al.*, *J. Immunol.*, 161(2):963-970 (1998); Picker, L.J., *et al.*, *J. Immunol.*, 145:3247-3255 (1990)). It is thought that specific chemokine receptors expressed on these lymphocyte subsets may interact with chemokines expressed in the areas mediating leukocyte
 15 activation, arrest, and transendothelial migration. It is therefore possible that CD4 subsets defined by the expression of certain adhesion molecules, may also express known, orphan or as yet undiscovered chemokine receptors that are important for trafficking of the lymphocytes into these sites. The work described herein relates to one such chemokine receptor that may be involved in the selective trafficking memory CD4
 20 and CD8 lymphocyte subsets to mucosal sites.

GPR-9-6 was originally chosen as a potentially interesting orphan chemokine receptor due to its strong phylogenetic linkage with other known chemokine receptors including CCR6 and CCR7. In Northern blot analysis, GPR-9-6 was found in thymus, indicative of some role in T cell development. The weak expression in spleen and blood
 25 may reflect the expression of GPR-9-6 on memory T lymphocytes and B lymphocytes. As GPR-9-6 is expressed by the majority of thymocytes, and these GPR-9-6^{+ve} thymocytes express all levels of TcR, GPR-9-6 is apparently expressed at all stages of T cell development. On exit from the thymus, GPR-9-6 must be down-regulated, as in the

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periphery only a small subset of CD4 lymphocytes and an even smaller subset of CD8 lymphocytes express GPR-9-6. In three-color experiments, GPR-9-6 is found predominantly on memory CD4 lymphocytes. Of greater interest, while the CLA⁺ memory CD4 lymphocytes (Picker, L.J., *et al.*, *J. Immunol.*, 145:3247-3255 (1990)) do not express GPR-9-6, a subset of the memory $\alpha 4\beta 7^{\text{high}}$ CD4 lymphocytes (Andrew, D.P., *et al.*, *Eur. J. Immunol.*, 26:897-905 (1996)) express this chemokine receptor. This may reflect a role for GPR-9-6 in the trafficking of lymphocytes to mucosal sites, or their effector action when there. While GPR-9-6 was clearly expressed on mucosal trafficking CD4 lymphocytes, GPR-9-6 transcripts were not detected in small intestine by Northern blot analysis. This may reflect the low numbers of the GPR-9-6⁺ CD4+ and/or CD8+ lymphocytes in small intestine tissue compared to thymus, where the majority of the cells are actively dividing GPR-9-6⁺ thymocytes. However, using the more sensitive technique of RT-PCR, GPR-9-6 transcripts were detected in small intestine but not in the brain. Interestingly, while GPR-9-6 and TECK transcripts are expressed in small intestine, GPR-9-6 or TECK transcripts were not detected in the colon by either Northern or RT-PCR analysis.

It is possible that factors present in the mucosal environment lead to the induction of GPR-9-6 on T lymphocytes as well as TECK expression. Cytokines present in T_H1/T_H2 environments induce expression of certain chemokine receptors, such as CCR4 on T_H2 and CXCR3 on T_H1 lymphocytes, as well as the production of the chemokines that bind these receptors (Bonicchi, R.G., *et al.*, *J. Exp. Med.*, 187:129-134 (1998); Sallusto, F.D., *et al.*, *J. Exp. Med.*, 187:875-883 (1998); Sallusto, F., *Science*, 277:2005-2007 (1997); Andrew, D.P., *et al.*, (1998); Zingoni, A., *et al.*, *J. Immunol.*, 161:547-555 (1998)). However, these conditions did not up-regulate GPR-9-6 expression on T lymphocytes. Also, attempts to induce expression of GPR-9-6 on activated umbilical CD4 lymphocytes with cytokines IL-1-18 or with TGF- β , previously shown to induce α E on T lymphocytes (Kilshaw, P.J. and Murant, S.J., *Eur. J. Immunol.*, 21:2591-2597 (1991)), failed to identify a cytokine that up-regulates GPR-9-6 expression. Therefore, the mechanism by which GPR-9-6 expression is controlled

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on CD4 lymphocytes is unclear. Upon activation via TcR cross-linking, expression of GPR-9-6 is down-regulated, as is the expression of chemokine receptor CXCR4 (Bermejo, M., *et al.*, *J. Immunol.* 28:3192-3204 (1998)). As TcR cross-linking mimics antigen presentation, we conclude that on entering a lymph node and encountering

- 5 APC's expressing antigenic peptide+MHC-II, that T lymphocytes will down-regulate chemokine receptors such as GPR-9-6. This will hold T lymphocytes in the lymph node, where T lymphocytes may mediate other immune functions such as B cell class switching through T:B cognate interactions.

- Out of all the chemokines tested only TECK (Vicari, A.P., *et al.*, *Immunity*,
 10 7(2):291-301 (1997)) acted as a chemoattractant for GPR-9-6/L1.2 transfectants, with 150 nM resulting in optimal chemotaxis. This falls into the range of 1nM-1 μ M for which other leukocyte chemokines are active. However, as we are using TECK that was generated by peptide synthesis, we cannot be sure that either post-translational modifications or further cleavage of TECK by factors outside the cell *in vivo* do not
 15 generate more active fragments, as is the case for CKB8 (Macphee, C.H., *et al.*, *J. Immunol.* 161:6273-6279 (1998)). TECK did not act as a chemoattractant for CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, CCR9 and CXCR1, CXCR2, CXCR3, CXCR4 and CXCR5 L1.2 transfectants. However, some weak activity of TECK on CCR3/L1.2 transfectants which was approximately 20% of the chemotactic activity observed with
 20 eotaxin-1 was detected. This activity was blocked by anti-CCR3 mAbs, though TECK did not act as a chemoattractant for eosinophils. Therefore, TECK is probably not a physiological chemokine for the CCR3 receptor. This result is not unprecedented, as in previous studies MIP-1 α has been shown to act as a chemoattractant for CCR4/HEK293 transfectants (Power, C.A., *et al.*, *J. Biol. Chem.*, 270:19495-19500 (1995)), but
 25 CCR4/L1.2 transfectants (Imai, T.M., *et al.*, *J. Biol. Chem.*, 272:15036-15042 (1997)). In further experiments, only the T cell lines that express GPR-9-6 were found to chemotax to TECK, while among primary cells TECK was chemotactic for only a small subset of CD4 lymphocytes. Presumably, these cells represent the small subset of CD4 lymphocytes that express GPR-9-6, as the chemotaxis was blocked by anti-GPR-9-6

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mAb 3C3. Additionally, only $\alpha 4\beta 7^{+ve}$ memory CD4 and CD8 lymphocytes chemotax to TECK, which would be the subset predicted to express GPR-9-6. TECK was originally described as a chemokine produced by thymic dendritic cell, whose expression is restricted to thymus and small intestine (Vicari, A.P., *et al.*, *Immunity*, 7(2):291-301 (1997)). Our Northern data confirms this observation and shows that the receptor for TECK, GPR-9-6, is also expressed at these sites. The expression of both chemokine receptor GPR-9-6 and its ligand TECK in small intestine and thymus predict a role for GPR-9-6 and TECK in T cell development and mucosal immunology.

In summary, the orphan chemokine receptor GPR-9-6 was shown to be expressed on the majority of thymocytes and on a subset of memory CD4 lymphocytes that traffic to mucosal sites. The selective expression of TECK and GPR-9-6 in thymus and small intestine imply a dual role for GPR-9-6, both in T cell development and the mucosal immune response.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

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